DIETARY AND CELLULAR MECHANISMS REGULATING HEPATOCYTE PROLIFEARTION AND CANCER

A DISSERTATION

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL

OF THE UNIVERSITY OF MINNESOTA

BY

JONATHAN M PLOEGER

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR A DEGREE OF

DOCTOR OF PHILOSOPHY

DR. DOUGLAS G MASHEK, ADVISOR

August 2017

Acknowledgements

I first wish to thank my advisor Dr. Douglas Mashek. You allowed a non-traditional student with a desire to challenge himself to join your lab. You worked with me and challenged me to make me the scientist I am today. I wish to also thank Dr. Mashek for his unwavering support of me, you are a tremendous scientist and a motivating mentor. Your push and support will never be forgotten.

I would also wish to thank Dr. Mindy Kurzer, my first advisor and the reason for me being here today. You too took a chance on a non-traditional student, providing me the opportunity of a lifetime to join the University of Minnesota as an NIH fellow. You also graciously allowed me to chase my passions in transitioning to Dr. Mashek's lab. I will forever be grateful to you.

I also wish to extend a thank you to my committee members Dr. Jeffrey Albrecht, Dr. Sabrina Trudo, Dr. Xiaoli Chen, and Dr. Kristen Nelson. Your guidance, expertise, and collaboration have proved invaluable over the past 4.5 years.

My gratitude and appreciation are also extended to my colleagues in the Mashek lab including Mara Mashek who provided extensive technical expertise and guidance. Your assistance during my tenure has been greatly appreciated and many of the experiments could not have been completed without you; thank you as well for your continued hospitality and genuine care. My appreciation extends to my lab mates, past and present, whom I consider not only my colleagues and mentors, but friends. Aishwarya, Charlie, Edith, Kenny, Mallory, Mostafa, and Salmaan; you are all tremendous scientists and better friends. Our relationships both professionally and personally helped carry me to the finish line and I hope

they will continue. Finally, I would like to thank the many tremendous undergraduates of the Mashek lab for their dedication and jubilant spirits: Coco, Christina, Ellen, Hosam, Joe, and Lauren you are fantastic young minds. Ultimately, I wish to provide a debt of gratitude to the funding sources that made this work possible: NIH T32 fellowship (5T32CA132670-05), NIH/NIDDK R01 – Albrecht (DK054921) and an Institutional Research Grant from the Healthy Foods Healthy Lives Institute.

Dedication

This work is dedicated to the most important people in my life: my friends and family. I share this work and success with you, because without you, it would not have come to fruition.

To my closest friends, thank you for being my support. It is a rare thing in life to find people that unpromisingly care for you and your happiness.

To my parents, thank you for always believing in me and for supporting your children in whatever they do. You have dedicated and sacrificed more for your children than could ever be imagined. The examples you set have helped shape me into the man I am today personally and professionally.

To Sandi, Greg, Mike and Julene, I could not have accomplished this work without you. Your enduring support of our family means the world to me and I consider myself beyond fortunate to have integrated into your lives.

To my brother and sister. I am so lucky to have you in my life. Your passions for what you do and your success are admirable and motivate me to better myself personally and professionally. I am beyond proud of you.

To my best friend, soulmate, and life-long companion. Nearly ten years ago you vowed to be my lifelong partner and to work together in a shared life. In nearly all ten of those years you have not only encouraged and supported my desire to pursue the unknown, but you have driven me to it. You are the embodiment of a spouse and a friend; you push me to be better and have unwaveringly supported myself and our growing family through years of my pursuit of a purpose. Your tireless care, your example as a wife, mother, sibling and friend, and your

strength are characteristics I strive to emulate. Thank you for being my rock, I love you.

And to my children. I began this process for you, hoping to be an example of pursuing your dreams and to interest you in science. Instead, you have taught me more than I could have ever imagined. Your spirits and genuine curiosity are a constant reminder to continue to ask questions. Your silliness and enthusiasm are reminders to not take life too seriously. Your unbiased love and acceptance are reminders to love unconditionally and to find acceptance in everyone and everything. You and your mother are what I look forward to most in each and every day. I love watching you grow and hope you never lose those attributes that make you such wonderful little people. Daddy loves you.

Abstract

Obesity is well-documented to promote the development of nonalcoholic fatty liver disease (NAFLD) including its more advanced stages such as non-alcoholic steatohepatitis, cirrhosis and hepatocellular carcinoma (HCC). While metabolic perturbations describing obesogenic progression from NAFLD to HCC have largely been investigated, our knowledge of the role lipolysis plays in this process is scant. This research project is aimed at understanding the role fasting lipid metabolism plays in pathologic features of carcinogenesis as well as synergistically combining with lifestyle factors to prevent obesity driven progression of NAFLD to HCC. To elucidate these features, we employed two seminal studies. The first study characterized the role of adipose triglyceride lipase (ATGL) in limiting a major cell cycle regulator, cyclin D1, and hepatocellular proliferation both in vitro and in vivo. We show that lipid catabolism via ATGL antagonizes cell proliferation. Additionally, we recapitulate these findings using a partial hepatectomy model to drive hepatocellular proliferation in vivo.

In the second study, we conduct a long-term carcinogenesis study that examines the role of dietary fat composition and lifestyle factors that promote fasting lipid metabolism. Animals were calorically restricted (CR) or exposed to regular endurance exercise. Using the hepatic carcinogen diethylnitrosamine (DEN), we show CR prevents hepatic tumor formation independent of dietary fat composition. RNA sequencing of non-transformed liver tissues revealed changes in metabolic pathways and reduced inflammation, cytokine production, stellate

cell activation and migration, and genes associated with liver injury and oncogenesis. Taken together, fasting hepatic lipid metabolism plays a significant role in mitigating proliferative effects often associated with overconsumption of calories. Furthermore, lifestyle factors that promote lipolysis in the liver robustly protected mice from developing tumors. Further investigation is warranted to define the molecular mechanisms ATGL plays in limiting hepatic proliferation as well as characterizing the role of ATGL and fasting in hepatic tumorigenesis.

Table of Contents

List of Tables

Chapter 3:

Caloric Restriction Prevents Carcinogen-Initiated Liver Cancer

List of Figures

Chapter 1:

An Overview of the Epidemiologic, Metabolic, and Cell Signaling Events Associated with Obesity Driven Hepatocellular Carcinoma

Chapter 2:

Cyclin D1 Regulates Adipose Triglyceride Lipase to Influence Mouse and Rat Hepatic Lipid Droplet Metabolism and Cell Proliferation

Chapter 3:

Caloric Restriction Prevents Carcinogen-Initiated Liver Cancer

CHAPTER 1

An Overview of the Epidemiologic, Metabolic, and Cell Signaling Events Associated with Obesity Driven Hepatocellular Carcinoma

Jonathan Ploeger wrote this chapter in its entirety

Global Impact of HCC

Introduction

The World Health Organization reports economic costs of treating cancer topped $1.16 trillion dollars in 2010, and projects worldwide incidence to jump 57% in the next 20 years (1). Of those cases, primary liver cancers are the fifth most common cancers worldwide and Hepatocellular Carcinoma (HCC) accounts for between 85-90% of primary liver cancers (2). Additionally, HCC is one of the most deadly forms of cancer worldwide with more than 700,000 cases diagnosed annually and greater than 600,000 of those cases resulting in death (3). Historically, developed countries including the United States have experienced low incidences of HCC, with more than half of all cases occurring in African and Asian countries. However, over the past decade incidence rates in the U.S. have tripled and are expected to continue trending upward in the coming years (4). Currently, HCC deaths in men are rising faster than any other cancer-related death in the United States (2).

Epidemiology of HCC & its Precursors

HBV/HCV & HCC

Hepatitis B (HBV) and Hepatitis C (HCV) viral infections remain the largest contributors globally to HCC respectively, contributing in the upwards of 84% of incident cases (3). As would be expected, HBV and HCV share a number of similarities and disparities as it pertains to the etiology of HCC. Both contribute to

chronic hepatic injury resulting in fibrosis and ultimately cirrhosis leading to pathogenesis of HCC. Furthermore, both HBV and HCV contribute to HCC through multiple oncogenic mechanisms including DNA methylation, proliferation promotion, increased ROS, and impaired autophagy (5). In addition, both viruses share similar risk factors that result in increased incident odds of HCC development: co-infection with HBV/HCV, alcohol use, male gender, HIV, diabetes mellitus, older age, African American race, esophageal varices, and smoking (3). However, whereas HCV is an RNA virus, HBV is a DNA virus, which allows its integration into the host genome where it can induce oncogenic mutations of host DNA (6). Furthermore, HBV accounts for the majority of worldwide HCC cases and does so in developing countries more often. A major contributing factor in HBV related HCC cases is aflatoxin exposure. One such report estimated the risk of liver cancer in HBV infected individuals exposed to aflatoxin to be 30 times greater than unexposed (7). Interestingly, in recent years incidence rates of HBV related HCC have declined in part due to effective vaccination programs and limited exposures to aflatoxin (8).

Conversely, developed countries have tended to associate the majority of HCC cases with HCV infection. For example, HCV once accounted for 40-60% of HCC cases in the United States despite only accounting for 30% of cases worldwide (9). However, safer procedures in blood transfusion and reductions in IV drug use have lowered incidences of HCV related HCC cases (3). Intriguingly, the reduction HCV has not resulted in an overall trend decrease in HCC. On the

contrary, cases of HCC have continued to rise over the past decade (4). This trend has led researchers to consider other etiologies of HCC.

NAFLD & HCC

First characterized in 1980 (10), non-alcoholic Fatty Liver Disease (NAFLD), as the name suggests, is a disease characterized by excessive fatty infiltration of the liver in non-alcohol users. If left unchecked, the overload of lipid increases inflammation of the parenchymal portal regions and organ damage, which leads to development of non-alcoholic steatohepatitis (NASH). The spectrum of NAFLD is the most common cause of liver disease in developed countries (11). Estimates from population sampling suggest 20-30% of Western country inhabitants have NAFLD (12). In addition, diagnosis of NAFLD is linked to greater mortality than the general population, with the majority of death resulting from liver diseases including HCC (13). Unlike incident rates of HCV/HBV in developed countries, rates of NAFLD/NASH continue to increase, largely as a result of their association with obesity and metabolic syndrome. Additionally, NAFLD/NASH as comorbidities with increasing age, the male sex, and type-2 diabetes (T2D) are well-established risk factors for the development of HCC (12). Although a recent prospective U.S. population study concluded a greater incidence of HCC from HCV related cirrhosis compared to NAFLD derived cirrhosis (14), the contrasting prevalence of HCV and NAFLD provides reason for concern. In fact, contrary to the previous study, a case control study found that when comparing cryptogenic cirrhosis (CC) to HCV related cirrhosis, CC patients were more likely to have obese BMI's, T2D, and elevated serum triglycerides

(TAG), all well-established risk factors for NAFLD and HCC (15). Taken together there is increasing data suggesting that NAFLD may become the primary source of HCC in the near future (2). Previously, it was thought NAFLD contributed to HCC through its progression to NASH which has a greater risk for progression to cirrhosis, with the first reported case of HCC derived from cirrhotic related NAFLD in 1990 (16). However, a number recent studies report HCC development in non-cirrhotic NAFLD (17).

In the past decade 116 cases of HCC have been histologically confirmed as derived from NAFLD without cirrhosis, which represents greater than 30% of all NAFLD resultant HCC cases (17). Additionally, the first reported case of NASH-associated HCC was reported as recently as 2005 (18). Increasing trends of advanced onset of HCC give reason for concern in developed countries including the U.S. For example, a case report conducted by Kawada et al. (19) using strict histological evaluation criteria to diagnosis NASH confirmed 75% of examined cases of NASH-related HCC did not show signs of cirrhosis. However, the authors report a potential for bias as the study population were all surgical resection patients. However, a number of other case reports have produced similar results leading to the conclusion that degree and severity of NAFLD/NASH risk factors may contribute to HCC (18, 20, 21).

Ultimately one of the greatest obstacles in gaining a deeper understanding of the role NAFLD plays in the development of HCC is obtaining accurate reporting. Because most patients present clinically at the point of cirrhosis, a point at which evidence of fatty infiltration has dissipated, it is suggested that NAFLD

associated cases of HCC are grossly under reported (17). Thus, while some prospective data suggests incidence of NASH related HCC is low or absent (22), the majority of evidence suggests NAFLD is primarily responsible for cases of CC. Such evidence was reported in a prospective study from the U.S. in which CC was the second leading etiologic factor to HCC only behind HCV. The study found clinical or histopathologic features of NAFLD in 50% of CC cases (23). Furthermore, a retrospective analysis concluded that features of NASH are more commonly found in HCC cases linked to CC (15). Despite HCV being an important etiologic factor in the global burden of HCC, NAFLD has established itself as a major risk factor in developed countries. Given the correlation between NAFLD and obesity, it is no surprise obesity is also strongly linked to HCC.

Obesity & HCC

Contrariwise to reducing trends in HCV, the United States is currently faced with an epidemic of obesity and obesity related diseases. In 2008, there were over 1.4 billion overweight individuals worldwide, with nearly 500 million of those being obese (24). Currently, the prevalence of obesity is greater than 35% in U.S. adult populations (25) and rates have increased between 2-3 fold over the past 3 decades in children (26). It is currently well-accepted that as obesity increases, so too does the risk of cancer in general and more specifically, liver cancer. Obesity has been estimated to be directly related to 14% and 20% of all cancer diagnoses in U.S. men and women, respectively (27). Obesity is particularly relevant to HCC because of its contributions to NAFLD. A prospective cohort of

over 28,000 Swedish men and women conducted by Wolk et al. (28) describes a 33% increase in the incidence of all cancers and a 3.6 times greater incidence of liver cancer in obese subjects compared to non-obese. In another large cohort conducted in the U.S., mortality rates from cancer paralleled increasing quintiles of BMI (29). In that same study liver cancer also showed a similar trend with a 4.52 greater risk of death in the highest BMI group compared to baseline quartile. Two recent reviews further corroborated these studies and confirm the association between obesity and HCC (30, 31).

Although generalized obesity has been demonstrated as an independent risk factor to NAFLD associated HCC, visceral adiposity appears to be a more accurate predictor. A number of recent studies, including a large prospective population study in Taiwan, have demonstrated an increased risk of NAFLD, HCC diagnosis and HCC reoccurrence in individuals with greater central adiposity (32–35). Visceral fat accumulation is thought to largely contribute to the pathogenesis of HCC due to the increased TAG burden placed on hepatocytes, particularly in the portal vein region. This increased intercellular fat burden is thought to cause excess endoplasmic reticulum (ER) stress which, leads to aberrations in cell signaling progressing the liver to NAFLD, fibrosis and ultimately carcinogenesis (36). Despite its contributions to fatty liver and HCC, obesity by itself does not fully explain NAFLD & HCC; nor can it solely account for the rapidly increasing prevalence of hepatic carcinogenesis seen in Western countries.

T2D & HCC

Along with obesity rates for typeT2D have increased exponentially over the past decade. Increased diagnostic rates of T2D and obesity in the U.S. from 1994-2010 have been well established (37). Interestingly, the results demonstrate that not only have both rates increased, but that they have increased in a paralleled manner. Furthermore, Information from the National Diabetes Statistics from the NIH estimates that 8.3% of the U.S. population has T2D (38). The rate of diagnosis for T2D alone is staggering, with total annual treatment costs reaching $174 billion (38). However, in accordance with the rise of obesity, T2D has also been linked to HCC (21, 39–42) and the association is further complicated when considering HCV (43). However, whereas obesity is a risk factor for NAFLD development and as a result an increased risk for HCC, T2D is thought to have a more unique relationship with HCC risk.

Loria et al. (44) propose a mechanism whereby genetic and lifestyle factors (e.g. obesity) lead to initial insulin resistance (IR). IR then contributes to NAFLD, which along with β-cell destruction leads to development of T2D. Finally, uncontrolled T2D in the setting of NAFLD increases risk for NASH and ultimately HCC. This theory falls in line with the “two-hit hypothesis” of NAFLD development where the first hit involves accumulation of TAG and steatosis as a consequence of IR. The second hit is long-term consequences of fat accumulation leading to necrosis and fibrosis which, ultimately results in the development of HCC (45). Indeed, evidence supports the importance of IR as a predictor of NAFLD independently of β-cell function (46, 47). Furthermore, a number of cross-sectional studies

support NAFLD as a risk factor for T2D (48, 49) with temporality supported by prospective studies (50–52). Consistent with the “two-hit hypothesis,” T2D derived from NAFLD has been associated with an increased risk for development of hepatic fibrosis (53) and ultimately has abidingly been linked to increased risk for HCC both in cirrhotic (39–42) and non-cirrhotic livers (54, 55).

The increased incidence of HCC in developing countries despite the positive strides made in controlling HBV/HCV outbreaks is alarming. The last decade has provided great insight in establishing strong connections between uncontrolled metabolic dysfunction and the development of HCC. Given the increasing evidence of metabolic perturbations in cancer cells, these epidemiologic relationships have drawn more recent attention in elucidating metabolic as well as molecular mechanisms that can explain the progression leading to NAFLD as well as in HCC.

Metabolic Alterations in HCC

Introduction

The elegant organization in the metabolism of nutrients to sustain cellular and organismal homeostatic function is quintessential in the sustainment of life. Despite the many intracellular redundancies aimed towards maintaining metabolic balance given a variety of external circumstances, many disease states require reprogramming of cellular metabolic function in order to thrive. These perturbations, while seemingly inefficient, often allow diseased cells to

bypass feedback regulation, alter cell signaling, and provide metabolic intermediates necessary to sustain survival even at the expense of the organism. The thought of cancer as a purely genetic disease was challenged as far back as 1924 by German biochemist Otto Warburg (56). However, it has not been until more recently that Warburg's theories as well as other metabolic alterations have been more extensively studied. These investigations have provided a litany of new information, hallmarking metabolic alterations in cancer development and proliferation.

Glucose Metabolism

Postprandial carbohydrate and gluconeogenic glucose is catabolized by target tissues, eventually generating 36 ATP for every molecule of glucose consumed through oxidative phosphorylation. In addition to ATP production, glucose can be converted to glycogen and stored for later energy production in times of fasting. These processes are well regulated by feedback mechanisms that limit influx of glucose when sufficient ATP is produced. However, the generation of ATP requires the cell to have sufficient cellular oxygen levels. In humans, if the cellular environment becomes hypoxic, pyruvate from glycolysis is fermented to lactate generating two ATP. In healthy cells, the replenishment of cellular oxygen inhibits further glucose fermentation in a process known as the Pasteur effect (57).

Despite these feedback mechanisms, Warburg discovered that cancer cells preferentially ferment glucose to lactate independently of intracellular oxygen

levels, or aerobic glycolysis, otherwise termed the Warburg effect (58). While this discovery led to many further metabolic discoveries in the context of cancer, Warburg's original theory was that cancer cells ferment glucose due to impaired mitochondrial function. This idea however, has been challenged (59) and in fact cancer cells rely on mitochondrial function to generate carbon intermediates for *de novo* lipogenesis (60) and amino acid synthesis (61, 62). Thus, while metabolic differences of healthy and cancer cells are fairly well characterized, they do not fully explain oncogenic transformation and resultant rapid proliferation.

Most multicellular organisms are offered an abundant nutrient rich environment. Therefore, without additional regulatory mechanisms most cellular environments would experience rapid and uncontrolled proliferation. To combat these deleterious changes, cells regulate nutrient uptake through growth factors. The requirement for growth factor presence to elicit proliferation, differentiation, and carcinogenesis has been well documented (63). Previously it was thought cancer cells must overcome growth factor dependence through genetic mutations that alter receptor-signaling pathway initiation. However, recent research has pointed to a "seed and soil" hypothesis, wherein overnutrition provides a microenvironment producing excessive levels of growth factors, which activate downstream targets such as protein kinase B (Akt) and mechanistic target of rapamycin (mTOR), ultimately producing a feed forward cycle resulting in uncontrolled proliferation (64). This mechanism has been well documented in a number of tissues (65–68) including the liver (66, 67). Despite the current model

that overnutrition contributes to rapid proliferation through altered growth factor presence, aerobic glycolysis is metabolically much less efficient than oxidative metabolism. Taken together, this means that aerobic glycolysis must 1) produce enough ATP to maintain cellular viability, and 2) provide metabolic products and byproducts used for continued proliferation to be considered a viable and sustainable alternative to oxidative metabolism.

Aerobic glycolysis is nearly 20 times less efficient regarding ATP production than oxidative metabolism. This process appears unsustainable for numerous reasons, one being maintenance of sufficient cellular ATP, as cells void of sufficient ATP often undergo apoptosis (69). Yet, cancer cells preferentially utilize fermentation and thrive despite its limitations. In demonstrating how this process maintains feasibility, Kilburn et al. (70) showed that the basal level of ATP necessary for cellular maintenance is far above that required for growth and division. Furthermore, increased glucose transport into cancer cells exceeds the bioenergetic demand for growth and proliferation achieved through aerobic glycolysis as demonstrated by high ATP/ADP ratios, even under circumstances of rapid cell division (71).

One clue into the ability of cancer cells to regulate the production of ATP considering increased glycolytic activity, lies in the observation that rapidly dividing cells also exhibit altered cell signaling. The phosphoinositide 3-kinase (PI3K) signaling pathway is perhaps the most well characterized pathway in glucose and growth. PI3K signaling controls and regulates amino acid metabolism in cancer via mTOR (72), lipid synthesis as well aspects of glucose

metabolism including glucose uptake (73) and increased activity of hexokinase (74) and phosphofructokinase (75). In addition, the penultimate step in glycolysis that catalyzes the generation of one molecule each of ATP and pyruvate is regulated by pyruvate kinase (76). Pyruvate kinase has four mammalian isoforms (L, R, PKM1, PKM2), of which the M1 isoform is expressed in adult tissues (71). Conversely, the M2 isoform is most widely expressed during embryonic development (77). However, what makes the M2 isoform necessary for embryonic growth and development is also ideal in cancer growth and proliferation (71). In contrast to the M1 isoform, PKM2 is regulated by tyrosine kinase signaling (78). In response to tyrosine kinase activity PKM2 is induced into a low activity state, diverting carbon to biosynthetic pathways and away from catabolic mitochondrial metabolism (78, 79). Furthermore, these studies have also demonstrated the necessity of PKM2 for continued cancer cell survival. Aerobic glycolysis has become the most well characterized metabolic perturbation in cancer. Warburg's seminal work has laid a foundation from which numerous important works have been crafted. Such studies have demonstrated a biological explanation for glucose sensitivity in several cancer cells of varying etiology and tissue specificity (80). Furthermore, evidence has grown indicating the cross-talk between signaling pathways and metabolic regulation important for glucose metabolism. More importantly, elucidation of aspects in glucose metabolism and cell signaling in cancer has established the overreaching importance of aerobic glycolysis in driving the biosynthetic aspects of other areas of metabolism.

Protein Metabolism

Recent years have explicated amino acids (AA) as multifarious molecules, contributing to cellular functions beyond cell signaling. Essential amino acids are defined as those which cannot be derived *de novo* and therefore, must be acquired from exogenous sources. Conversely, non-essential amino acids (NEAA) are those which can be attained from exogenous or endogenous sources. Interestingly, there is growing evidence that besides their role as building blocks of proteins and polypeptides, certain AA play a larger role than once thought in the regulation of key metabolic pathways that are necessary for cellular maintenance, growth, and proliferation; they are called functional or conditionally essential AA and include arginine, cysteine, leucine, proline, tryptophan, and most relevantly to cancer glutamine (81).

Alterations in glucose involved metabolism and cell signaling in cancer cells noted by Warburg and others has provided important insights to other areas of nutrient metabolism in oncogenesis. The cellular demand for glutamine in cancer was established as early as 1955 (82). However, it has not been until more recently that metabolic and biochemical studies have demonstrated that increased glycolytic function cannot by itself sustain growth and proliferation and indeed requires other nutrients such as AA. The amino acid glutamine contributes to core metabolic tasks of proliferating tumor cells. For example, glutamine provides substrate for cellular bioenergetics (83), supports defenses against oxidative stress (83, 84), and complements glucose metabolism in the production of biomass (85). Collectively, the diverse contributions of glucose and

glutamine metabolism paint a more complete picture of oncogenic growth and proliferation.

Conventionally glutamine's contributions to cellular function are limited primarily to contributing to nitrogen stores. However, during rapid growth and proliferation the cellular demand may exceed synthesis by 10-fold, making glutamine a conditionally essential AA (86). Growing cancer cells require the generation of NEAA for the biosynthesis of proteins and nucleotides as well as for continued generation of ATP (85). Much of this demand is met through the marked increase in glutaminase, the enzyme that catalyzes glutamine to glutamate (87). The nitrogen generated from the glutaminase reaction is allocated to various pools for the generation of NEAA; alanine and aspartate are particularly generated and have been targeted as biomarkers of cancer (88). Both NEAA are readily utilized in protein synthesis; aspartate also contributes to nucleotide synthesis as well as provides electron donation to complex I of the electron transport chain leading to ATP generation through its contribution to the malate-aspartate shuttle (76, 86). Glutamine also directly contributes to purine and pyrimidine synthesis (89). The γ-nitrogen from glutamine are added to growing purine rings as well as contribute to the conversion of xanthine monophosphate to guanosine monophosphate (86).

Reactive oxygen species (ROS) are produced from normal cellular respiration and metabolism, but can have deleterious consequences when homeostasis is not maintained. One consequence of increase ROS generation is mitogenesis (90). However, another consequence of increased ROS is the induction of

apoptosis (91). Therefore, cancer cells like their non-transformed counterparts must balance the production of ROS to survive; glutamine metabolism plays a role in maintaining this balance. Besides aspartate and alanine, cysteine is generated from the glutamate pool derived from glutamine metabolism (76). Cysteine is derived from an antiporter system in which, glutamate is exported from the cell while cysteine is imported. Following importation, cysteine is converted to cysteine using intercellular glutamate, ultimately producing glutathione (GSH), a major balancer of ROS that has been targeted for cancer therapy (92).

While glutamine metabolism is essential to cancer cells in the generation of nucleotides, proteins and antioxidants, the metabolic efficiency of glutamine exceeds these demands. Additionally, since the PKM2 isoform drives fermentation of glucose, alternative metabolites are required for cellular energy. Therefore, glutamine sustains the mitogenic environment by providing ATP and reducing equivalents. After the conversion of glutamine by glutaminase, glutamate acts as a respiratory substrate through further conversion of its carbon backbone to α-ketoglutarate. Once committed to glutaminolysis through the TCA cycle, glutamate has a number of fates and may be oxidized to malate and shuttled out of the mitochondria to form pyruvate which, can be shuttled back into the mitochondria to form acetyl-Coenzyme A (Ac-CoA), making glutamine a major anaplerotic substrate in cancer metabolism (85, 93). Important to note of this process is the contribution to cellular energetics; as α-ketoglutarate is converted to succinyl-CoA, NADH is produced which is utilized by complex I of

the electron transport chain, ultimately producing ATP. Furthermore, the conversion of malate to pyruvate by malic enzyme produces NADPH, reducing equivalents used in growth, proliferation, and energy production (85). However, DeBerardinis et al. (89) have noted that much of glutamine's carbon is exported from the mitochondria as lactate, adding to the pool from aerobic glycolysis (89). Finally, glutamine may also be exported from the mitochondria as citrate, which can be converted to oxaloacetate, then malate, and finally pyruvate generating NADPH while contributing to anaplerosis or the citrate may be used for another metabolic hallmark of cancer cells: *de novo* lipogenesis (60, 93). Nonetheless, under hypoxic conditions this process can be reversed through what is termed reductive glutamine metabolism, via isocitrate dehydrogenase-1 (IDH-1) (60).
In addition to metabolic contributions glutamine has been established as a driving force in cancer cell survival and growth. In fact, cellular glutamine uptake has recently been shown to be the rate-limiting step necessary for the EAA and growth factor regulation of mTOR complex 1 (mTORC1) (94). These findings are unsurprising given that glutamine and ATP levels are inversely related. Therefore, as ATP levels decrease, AMPK levels rise. Increasing levels of AMPK trigger mediators of autophagy (95). Furthermore, AMPK phosphorylates tuberous sclerosis complex 2, inhibiting mTORC1 and stunting growth and proliferation (95). Conversely, the presence of growth factors and a nutrient rich environment, particularly glutamine, drives the activation of mTOR complex 2 (mTORC2) and mTORC1, respectively (94, 96). The activation of mTORC1 leads to downstream phosphorylation and activation of ribosomal protein S6 kinase

(S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which stimulates translation, ribosomal biogenesis, and cell growth (96).

While it has been well-established that cancer cells massively increase utilization of various macro and micro-nutrients in carbohydrate and protein metabolism, increasing evidence supports the notion that nutrient excess drives the oncogenic activation (97). Collectively, glucose and glutamine metabolism work synergistically to regulate a number of processes required of proliferating cells. Interestingly, while glucose and glutamine metabolism drive various aspects of growth and proliferation individually, the metabolic processes of each also contribute to the sustainment of each other. This fact remains true in an additional hallmark of cancer metabolism: *de novo lipogenesis*.

Lipid Metabolism

Introduction

Cancerous cells must maintain metabolic efficiency to maintain growth and proliferation. Given the reliance on amplified uptake and subsequent altered metabolism of glucose and glutamate, it is no surprise that a need for cataplerotic events is necessary for maintaining oncogenic metabolic homeostasis. *De novo* lipogenesis (DNL) is one such cataplerotic event that is increased in cancer (98). However, while oncogenic alterations of metabolism are predominantly universal from tissue to tissue and etiology to etiology, lipid metabolism has a unique role in these respects as it relates to oncogenesis. It is well understood that the liver is a major organ for FA trafficking in the human body. FAs derived within the liver

primarily arrive from FFA uptake, chylomicron remnant uptake, and/or DNL. However, cancer cells favor amplification of *de novo* synthetically derived FAs over exogenous uptake (98). Unlike aerobic glycolysis and glutamine metabolism which are universal perturbations in oncogenic metabolism, DNL provides a mechanistic explanation to the recently increasing development of HCC via NAFLD. Previous studies have demonstrated that DNL accounts for ~8% of hepatic FAs during fasting, but increase to 27% post-prandially (99). Interestingly, intrahepatic TAG derived from DNL mirrors post-prandial levels in patients with NAFLD (100) and liver cancer (101).

De Novo Lipogenesis in Cancer

Regardless of the source of derivation, FAs must be “activated” by long-chain fatty -acyl-CoA synthetase (ACSL) by ligating acyl-CoA to FA in forming FA-Acyl-CoAs for sequestration into the cell and for access into the bioactive pool (102). Several isoforms of ACSL exist with ACSL3 and ACSL5 being most prominent in hepatic tissue (103) and have been identified as essential to lipid droplet (LD) formation in HCC cell lines (104). Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) sequentially carry out the committed steps of DNL converting acetyl-CoA to malonyl-CoA and FAs respectively. However, ACC has cytosolic and mitochondrial isoforms, ACC1 and ACC2, respectively. While ACC1 is most commonly associated with DNL, its targeted hepatic ablation increases ACC2 activity accordingly, making it necessary but not sufficient in maintaining DNL (105). Malonyl-CoA produced by ACC1/2 provides substrate for elongation by

FAS that terminates upon the generation of palmitate (16:0) (106). Further elongation of hepatic 16:0 is primarily carried out via long-chain fatty acid elongase (ELOVL) 5, which produces mostly oleate (18:1) (107). Additionally, stearoyl-CoA desaturase (SCD)-1 generates palmitoleate (16:1) and oleate (18:1), while longer chain FAs are derived from the action of other elongase enzymes such as ACSL. The ACSLs are responsible for eventual incorporation of FAs into TAG and phospholipids; their dysregulation is an important indicator of altered DNL realized in HCC (108).

Increased TAG formation is known to accompany DNL. Dysregulation of lipid metabolism resulting in excess TAG within hepatic LDs is a hallmark of NAFLD (109), independent of liver injury (110), and its progression to HCC (108). The initial step of *de* novo TAG synthesis is carried out by the enzyme sn-1-glycerol-3-phosphate acyltransferase-1 and produces lysophosphatidic acid (111). This enzyme is a key contributor to excessive hepatic TAG accumulation and its deletion has been associated with reduced susceptibility to HCC (112). Conversely to newly synthesized FAs, sn-1,2-diacyglycerol acyltransferase-2 (DGAT2) esterifies diacylglycerol to TAG from *de novo* sources whereas DGAT1 esterifies FAs from exogenous sources. Overexpression of DGAT2 is sufficient to drive steatosis independently of IR (113). Moreover, these enzymes have been targeted in cancer therapy and inhibition of the DGATs has proved effective in ablating TAG synthesis in the human liver cancer cell line HepG2 (114). In addition to the DGATs, monoacylglycerol acyltransferases have also been implicated in NAFLD (115).

Clinical Implications of Lipid Metabolism and HCC

Clinically important, a single nucleotide polymorphism (I148M) of the gene patatin-like phospholipase domain containing A3 (PNPLA3) has been identified as a predictor of NAFLD (116) and, importantly, I148M has also been implicated as an increased risk for fibrosis as a result of steatosis and for HCC independently of fibrosis (117, 118). PNPLA3 encodes the protein adiponutrin, which is in the same family as ATGL. Given TAG accumulation and subsequent NAFLD, I148M has been presented as a loss of function single nucleotide polymorphism producing reduced lipolytic activity (119). However, more recently Kumari et al. (120) demonstrated no reduction in TAG hydrolysis with adiponutrin polymorphism. Instead, their evidence reported increased production of PA and subsequent accumulation of TAG in hepatic LDs, akin to the activity of the acyltransferases. Moreover, recent lipidomic analysis of the human hepatocyte derived cellular carcinoma cell line HUH7 revealed that compared to its wild type (WT) counterpart, I148M induced TAG formation from existing unlabeled FAs whereas WT PNPLA3 incorporated more TAG from *de novo* (121). Conversely to the Kumari report (122), however, no difference was found in acyltransferase activity (121). In addition, WT PNPLA3 was found to enhance remodeling of TAG with 18:1, which resulted in a greater ability for hydrolysis, events defective in I148M (121). Adding to confusion, each recent report has demonstrated a different role for PNPLA3 in the development of NAFLD and HCC with each report refuting the findings of the previous study. Collectively, there is a definite

association for PNPLA3, but much is left yet to confirm the mechanism whereby the single mutation of the gene contributes to these liver diseases.

Oncogenic Lipid Metabolism Cell Signaling

With greater recognition of lipogenesis in cancer, there has been an increased focus on defining the cell signaling mechanisms that lead to cancer initiation. Because oncogenesis has been described as a consequence of overnutrition, and because DNL activity hinges nutrient status and insulin sensitivity of the organism, it should be no surprise that mTORC1 cell signaling cascades play a central role in oncogenic lipid metabolism dysregulation. mTORC1 had been implicated in adipogenesis by phosphorylating 4E-BP1 leading to its dissociation from eukaryotic translation and initiation factor 4E. This process has been shown to increase sensitivity to diet induced obesity and promotes IR (122) and is mediated by 4E-BP1 induction of peroxisome proliferator-activated receptor (PPAR)-γ (123). However, increases in phosphorylated S6K are also noted in adipocytes lacking the mTORC1 negative regulator, tuberous sclerosis complex I, suggesting a role for S6K in adipogenesis (123).

The liver produces TAG from *de novo* sources in the fed state in preparing energy reserves during periods of fasting. During the fasting period, ketone bodies are produced in the liver as a consequence of acetyl CoA levels exceeding the capacity of the TCA cycle (124). However, with IR, ketone body production is increased during fed conditions. This can mechanistically be explained through mTORC1 signaling cascades as it has been demonstrated

that inhibition or tuberous sclerosis complex 1 leads to decreased ketone body production by promoting nuclear localization of nuclear receptor co-repressor 1, which, in turn increases its inhibitory binding of the major ketone body transcription factor PPARα (125); mTROC1 phosphorylation of S6K2 mediates these effects on PPARα (126). In addition to inhibition of PPARα activity, a major transcription factor for fatty acid oxidation, mTORC1 also promotes lipogenesis by suppressing lipolysis via inhibition of adipose triglyceride lipase (ATGL) (127). Therefore, it is no surprise recent publications have highlighted the role of mTOR in hepatic lipogenesis.

The first work to identify mTORC1 in hepatic lipogenesis demonstrated that gene transcription for DNL was dependent on activation of sterol regulatory binding protein 1 (SREBP-1c) and that its activation was dependent on Akt mediated activation of mTORC1 (128). Furthermore, the induction of SREBP-1c by mTORC1 was inhibited in the presence of rapamycin (128). Successive studies employing gene expression profiling identified S6K as the required intermediate for SREBP1c activation *in vitro* (129). Calvisi et al. (130) demonstrate ribosomal protein S6 (S6), a target of S6K, is necessary for posttranscriptional SREBP1. Supporting this, of more recent work has demonstrated that transcriptional regulation of an isoform of SREBP, SREBP1c, is not dependent S6K, while posttranscriptional processing is (131). Further explaining SREBP regulation via mTORC1, research conducted by Peterson et al. (132) demonstrated that mTORC1 phosphorylates and inactivates lipin1. Under basal conditions lipin1, a phosphatidic acid phosphatase translocates to the nucleus leading to nuclear

eccentricity and thus suppresses transcriptional activity of SREBP1c (132). However, mTORC1 phosphorylates lipin1, relegating it to the cytoplasm, which induces transcription of SREBP1c and increases target gene mRNA levels (132). However, completed works linking mTORC1 to SREBP1c and increased DNL in cancer give rise to two important points of emphasis that require further elaboration. First, our understanding of the mechanism by which S6K and lipin1 regulate SREBP1c remains largely unknown. Secondly, while mTORC1 has been demonstrated necessary for SREBP activity, recent works have highlighted that it is not sufficient to induce activity and requires mTORC2 for sufficient phosphorylation of Akt and thus downstream DNL effects (133), especially in hepatic tissue (134). SREBP1 activated by mTORC1 and mTORC2 has clearly been identified as in important regulator of DNL in hepatic and other cancers and has recently been shown to play a predictive role in HCC pathological outcomes (135). It should therefore, be no surprise that many targets of DNL, including SREBP1 have become targets for cancer therapy.

Lipid metabolism from *de novo* sources is an essential metabolic process in cancer cell signaling, growth, and proliferation (136) as a downstream consequence of increased aerobic glycolysis and anaplerotic input from glutamine metabolism. As a result, several studies have examined lipogenic enzymes in searching for potential therapeutic susceptibilities in oncogenesis. ATP citrate lyase (ACL) links glucose metabolism to FA metabolism by converting citrate to acetyl-coA for DNL and other metabolic processes. Inhibition of ACL via siRNA has been demonstrated to reduce lipid synthesis and cell cycle

progression in an adenoma cell line from glucose, but not acetate (137) suggesting the necessity of the enzyme in cancer cell survival via DNL. Furthermore, chemical inhibition of ACL was shown to be antineoplastic in vivo (137).

Upon acetyl-CoA generation, ACC carries out the rate-limiting step of DNL by carboxylating acetyl-CoA to generate malonyl-CoA. Being the rate-limiting enzyme of DNL, ACC has been shown to be markedly upregulated in HCC (138) and its knockdown has been shown to induce apoptosis in certain cancers (139). Additionally, inhibition of ACC1 by activation of the AMPK pathway has been shown to antagonize mTOR activity and deter oncogenic proliferation via inhibition of the cyclins, including cyclin D1 (140). However, other cancers growth has been enhanced with ACC1 silencing (141), making its target for therapeutic intervention controversial. Nevertheless, chemicals such as Soraphen A have been used to deter cancer survival by blocking fatty acid elongation (142).

Independent of malonyl-CoA generation, fatty acid synthase (FASN) primarily functions to generate palmitate from malonyl-CoA, but can generate FA directly from acetyl-CoA as well; unsurprisingly, it is another enzyme upregulated in cancer (143). Its mechanism for overexpression has been hypothesized to be a result of increased SREBP1C transcription and/or through reduced proteasomal degradation (143). As would be expected, targeted inhibition of SREBP1C reduces transcription of FASN, leading to abrogation of neoplastic characteristics (144). Furthermore, therapeutic targets of FASN have also been established as oncogenic inhibitors (145).

The function of FA is dependent upon chain length and saturation; ELOVL and SCD carry out these functions respectively and are important in oncogenic risk. ELOVL6, one of seven ELOVL isoforms is increased in NASH, a major risk factor for HCC development (146). However, other elongases such as ELOVL5 have been found to be increased in response to androgen treatment, but were not enriched in prostate cancer samples suggesting the isoforms activity may be a secondary response to alterations in androgen signaling rather than driving oncogenic processes (142). Desaturation post elongation by FASN is carried out by SCD; it is also upregulated in numerous cancers (147).

ACSL has also demonstrated its necessity in maintaining hepatic lipogenesis in neoplastic cells by promoting LD formation via FA activation (104). Furthermore, overexpression of ACSL4 deterred inflammatory signals in colon adenocarcinomas, leading to increased survival (148). Conversely, inhibition of ACSL4 has been demonstrated to be effective in reducing breast tumor growth *in vitro* (149) suggesting it may be an effective therapeutic target in cancer. Indeed, several drugs such as the thiazolidinediones have successfully inhibited rat ACSL4 (150).

Once synthesized, FAs are trafficked into several cellular processes including membrane synthesis, cellular energetics, and storage. FAs incorporated into TAG are stored as ER derived organelles, intracellular LD. While several signaling pathways induce LD formation the precise mechanism by which they are generated is still contended. However, the most generally accepted mechanism involves the LD budding from the ER domain. In this theory, neutral

lipids accumulate between the bilayer of the ER eventually leading to a lipid dense ER bud which, eventually separates from the ER forming a nascent LD (151). Healthy tissue including liver contains few small LDs. However, increased numbers of LD's occurs in NAFLD (109) as well as HCC (108, 152) and other cancers (153). To meet hyperplastic and hypertrophic changes in LD's, many physiologic changes must occur including increased lipid production (which is met exogenously and, to a greater extent, in cancer from DNL) and reduced turnover from lipolysis. With TAG making up the largest component of the LD, it would be inferred that activity of ATGL, the rate limiting enzyme of TAG turnover, would be reduced. Indeed, studies have demonstrated activation of the mTOR pathway blunts lipolysis by hindering the activity of ATGL in adipocytes (154), whereas rapamycin treatment leads to reduced LD's and subsequently eicosanoid production (155). In addition, several proteins associated with LD structure and accessibility encourage growth and inhibit turnover of oncogenic LD's.

Lipid Droplet Proteins and Hepatic Disease

Perilipins (Plin) are a family of LD surface proteins. The most well characterized LD proteins, their presence is dependent on tissue distribution (156), stage of growth (157, 158), and ligand preference (159). Plin2, and Plin3 are the major hepatic LD Plin proteins (160). However, perilipin (Plin1) and Plin2 have been found to be expressed in development of hepatic steatosis (161, 162) neoplastic steatogenesis (163), respectively. Plin1 contributes to TAG sequestration in LD

through physical interaction with the protein CGI-58 (164), which is a co-activator of ATGL and when bound can increase the lipases activity nearly 20-fold (165), whereas KD of CGI-58 has been shown to lead to steatosis and fibrosis through a reduction in lipolytic activity (166). Conversely, lipolytic signaling through protein kinase A (PKA) leads to dissociation of Plin1 from CGI-58 allowing for interaction with ATGL (164). Plin2 has also been suggested to deter lipid hydrolysis by excluding ATGL from the LD surface (167). Moreover, obese IR rats fed diets rich in conjugated linoleic acid demonstrated significant improvements in hepatic steatosis compared to control fed mice in part through decrease in Plin2 expression (168). Additionally, genetic ablation of Plin2 protected mice from diet induced obesity and NAFLD (169). Further supporting this role, Plin2 and not Plin3 has been demonstrated necessary for hepatic regeneration, in part through its ability to increase lipid content required of sustaining hepatic proliferation (160). While Plin3 has not been shown to be necessary in regenerating liver, its reduction was shown to improve hepatic steatosis while improving murine glucose homeostasis (170). Recent studies have further implicated LD proteins in hepatic cancer in demonstrating that pharmacologic induction of ER stress in HUH7 cells resulted in an increase of several lipid metabolism enzymes and LD proteins including Plin2 (171). This finding supports the presence of other LD proteins beyond the Plin family as having altered expression in NAFLD/NASH as well as proliferating and neoplastic tissue.

The Cell death-inducing DNA fragmentation factor 45-like effector (CIDE) is another family of proteins that associate with LDs. CIDE proteins function as apoptotic proteins (172), but also play an important role in LD maintenance (173). Prior to 2003 two human isoforms of CIDE had been identified (CIDE-A and CIDE-B), whereas three isoforms existed in mice (CIDE-A, CIDE-B and CIDE-C). However, Liang et al. (172) identified CIDE-C, the human homolog to CIDE-C and its expression was increased in HEPG2 cell lines compared to adipose tissue samples and other cancer cell lines despite lower basal expression in hepatic tissue. Controversially, CIDE-C expression has also been demonstrated to be reduced in patients diagnosed with HCC and OE induced apoptosis (174). However, patient identification does not describe etiology of HCC, warranting further investigation as to whether derivation of HCC from HCV or NAFLD displays differences in LD protein expression. Recently, Plin1 has been revealed to physically interact with CIDE-C, an interaction responsible for LD formation through lipid exchange between LD's (LD fusion) in mature adipocytes (175). Additionally, CIDE-A has been shown to share similar function in both brown and white adipose tissue (173).

Where Plin1 deters lipolysis by sequestering the ATGL co-activator CGI-58, G0/G1 switch gene-2 (GOS2) also antagonizes ATGL-mediated lipolysis by anchoring ATGL to the LD surface, prohibiting hydrolytic activity in adipocytes (176, 177) and in hepatocytes (178). The increased TAG content of hepatic LDs as a result of GOS2 OE also appears to be important in cancers (179) including

hepatic carcinoma as GOS2 was found to have increased expression on microarray and RT-PCR screens of patients diagnosed with HCC (180). Although oncogenic *de novo* lipogenesis is well understood, it is only one aspect of an elegant system of human metabolism. More interest is still needed in teasing apart other aspects of lipid metabolism in oncogenesis. Oncogenic signaling directly deters lipolysis through mTOR inhibition of ATGL (154) and indirectly impacts lipolysis by increasing LD proteins known to inhibit ATGL (181). Conversely, β-adrenergic signaling is known to drive catabolic signaling of lipid metabolism, antagonistic to many aspects of mTOR regulated anabolic signaling. Stimulation of β-adrenergic receptors is known to increase cyclic AMP levels leading to activation of PKA, which triggers lipolysis by stimulating ATGL. However, insulin disrupts this signaling cascade (182), the first step in mTOR signaling. Interestingly, stimulation of PKA also phosphorylates Plin1 (183), causing it to dissociate from CGI-58 (164) and stimulate the rate limiting process of lipolysis. Ultimately, the current evidence points to antagonistic roles in β-adrenergic signaling and mTOR signaling as it pertains to LD protein function and location and lipid turnover. However, the mechanisms explaining such differences are still very contentious and require further investigation.

Hepatocyte Proliferation

Cell Cycle

When placed under stimulatory conditions either *in vitro* or *in vivo,* mammalian cells begin the process of cell growth and proliferation, often referred to as the

cell cycle. Upon initiation by growth factors and other mitogenic stimuli, cells must decide to exit a quiescent state, also termed G_0, or to actively enter the cell cycle. Cells entering the initial phase of the cell cycle, the G_1 phase, must immediately begin to prepare the cellular environment to undertake the momentous effort required for cell replication. Part of this preparation involves the accumulation of biomass required for the doubling of other macromolecular components necessary for cell proliferation. This initial accumulation of cellular constituents is referred to as cell growth, a term often incorrectly applied synonymously with proliferation. During the processes entailed in the G_1 phase, proliferating cells must make critical decisions regarding sustained growth or quiescence. Cells completing the growth phase must then pass a critical checkpoint, termed the restriction point or “R point,” before transitioning to the synthetic or S phase. While several checkpoints exist throughout the process of the growth and proliferation, the R point is often considered the rate limiting step of the cell cycle because passing this checkpoint ultimately commits the cell to completion of the cell cycle (184). During S phase, genomic material is doubled, creating two sets of DNA; this process generally requires 6-8 hours for completion, however, this process duration varies drastically among cell types (185). Prior to cytokinesis mammalian cells, for yet ill-defined reasons, dedicate 3-5 hours in preparation for entrance into mitosis (the M phase) (185). The M phase culminates with the generation of a new daughter cell which can re-enter G_1 or in the presence of cell cycle inhibitors may revert into a quiescent state (fig. 1).

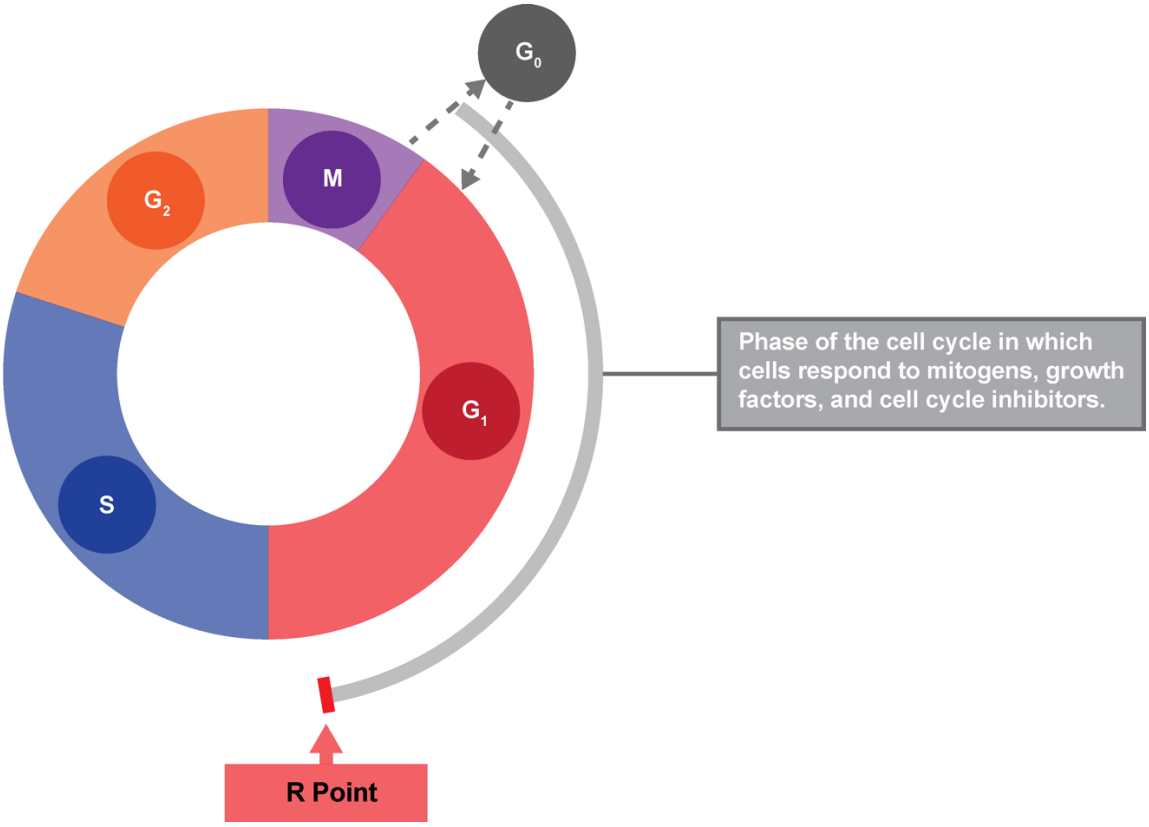


Figure 1. Visual representation of the cell cycle.

Upon completion of the M phase or quiescent cells in the G_0 phase stimulated by mitogens or growth factors, enter the G_1 phase of the cell cycle. Near the end of the G_1 phase, cells reach the restriction or "R" point. Cells bypassing this critical checkpoint continue to the S phase and are committed to cell cycle completion. Cells not meeting the requirement for continuation are delayed until corrections/repairs can be made or continuation is halted completely. Cell then double their DNA content during the S phase before entering the G_2 phase. The subsequent and final M phase or mitosis is where cells halve newly doubled DNA content through prophase, metaphase, anaphase, and telophase resulting in two complete sets of genetic material and cellular material. Attached to telophase, cytokinesis splits the cytoplasm of the cell resulting in a newly formed daughter

cells. These cells may then renew the cell cycle or enter G_0 until stimulated again.

Cell Cycle Proteins

The cell cycle process is highly regulated, ensuring the integrity and viability of rapidly growing and dividing cells. Regulating this process, cyclin family of proteins (cyclin's), cyclin-dependent kinases (CDK's), and cyclin-dependent kinase inhibitors orchestrate the transition between critical points of the cell cycle. Cells entering the cell cycle may do so from either renewed entry following completion of the M phase or upon stimulation of cells residing in the G_0 phase. Quiescent cells enter the G_1 phase as a consequence of cyclin C forming a complex with CDK3 (186). The G_1 phase is primarily governed by the activation of the D type cyclins and their subsequent binding to CDK 4 or CDK6 (187). Of importance, cyclin D1 directs the transition through the R point of the cell cycle. In the nucleus of mitogen stimulated hepatocytes, cyclin D1 forms a complex with CDK 4-6 downstream of mTOR (188). This newly formed cyclin D1-CDK 4/6 complex leads to the phosphorylation of tumor suppressor protein Rb, causing it to dissociate from E2 factor (E2F) (189). Now dissociated, E2F drives transcriptional activation of genes involved in promotion of G_1-S transition (190); cyclin E is one such gene activated through this process.

Culminating in the transition past the R point, cyclin D type proteins share an inverse relationship with cyclin E. At the initiation of the S phase, cyclin D1 is phosphorylated at Thr-286 by Glycogen synthase kinase 3β (GSK3β) (191),

flagging it for cytosolic export and subsequent proteasomal degradation (192). Once initiated by E2F's transcriptional activity, cyclin E forms a complex with CDK2 (193) completing the transition into, and through much of the S phase. Navigation through the S phase is additionally mediated by cyclin A. As with the fate of cyclin D1, the S phase triggers the degradation of cyclin E, preventing re-replication of DNA material (194). This frees CDK2 to reform an activation complex with cyclin A, leading to the completion of the S phase (195), while the G_2 phase is guided by formation of cyclin A-CDK1 complexes (195). The final M phase of the cell cycle is initiated by the formation of cyclin B-CDK1 complex and its ubiquitination signals termination of the final phase and completion of the cell cycle (196).

Cyclin D1 Effects on Metabolism

Cyclin-CDK complexes play a central role in coordinating expeditious and flawless transitions throughout the cell cycle. However, recent work has implicated the regulatory governance of cyclin proteins in macronutrient metabolism. This is not surprising as protein, carbohydrates, and lipids play integral roles in sustaining structural and energetic demands of cell replication. Cyclin D1 has been one of the most well defined cyclin proteins in metabolic regulation and has been implicated in directing glucose, lipogenesis, and amino acid metabolism in the liver.

In the fasted state, GSK3β leads to cyclin D1 retention in the cytoplasm and its subsequent degradation (192). Conversely, in the presence of insulin, Akt

phosphorylates and inhibits GSK3β and promotes a significant increase in nuclear cyclin D1 (192, 197). This increased presence of cyclin D1 has recently been shown to increase transcription of general control non-repressed protein 5, which leads to increased acetylation of PGC-1α and repression of gluconeogenesis independent of cell cycle progression (198).

During periods of fasting, amino acid catabolism in part, serves to provide carbon intermediates in glucose production. Produced glucose can then be secreted and transported to tissues to meet energetic demands. During nutrient sufficiency, amino acids serve as anaplerotic substrate as well as building blocks for nucleotide synthesis of rapidly dividing cells. Withdrawal of NEAA leads to undetectable levels of cyclin D1 and ectopic overexpression of D1 in the same conditions rescues proliferative effects (199). Moreover, essential amino acids have a more profound effect on cyclin D1 expression than do their non-essential counterparts (198), collectively demonstrating the interplay between amino acid sufficiency and cyclin D1 in the cellular replication process.

One of the most profound influences cyclin D1 has demonstrated on metabolism is its effects on lipogenesis. Cyclin D1 has been demonstrated to be elevated during in NAFLD progression in animals (200) and metformin's clinical efficacy has in part been demonstrated through its inhibition of cyclin D1 (201). However, despite its transcriptional and translational activity associated with increased steatosis, cyclin D1 has been shown to impede lipogenesis. In the presence of glucose, carbohydrate response element binding protein transcriptionally regulates hepatic lipogenesis (202–204). Cyclin D1 inhibits lipogenesis through

its repression of carbohydrate response element binding protein in a CDK4 dependent manner (205). However, in addition to its inhibition of lipogenesis, cyclin D1 also inhibits PPARα through a CDK4 independent manner (206). Given lipolysis regulates PPARα (207), which is well-established for its role in promoting fatty acid oxidation (109), these findings establish a unique link between cyclin D1, lipid metabolism and hepatocyte proliferation.

Current Objectives

Cancers are multiform in their etiology and metabolic alterations. Metabolism is a vastly complex system with much cross-talk and feedback mechanisms ensuring vitality of the host. Cancers utilize the foundation of these innate designs to ensure their own survival at the expense of the organism. Many challenges exist in curbing such perturbations: multiple enzymatic isoforms, different tissue distributions, varying reliance of specific enzymes and proteins from cancer to cancer, bridging of macronutrient metabolism and varying roles of proteins as anti/pro carcinogenic are but a few obstacles researchers and clinicians face in finding therapeutic and lifestyle interventions that successfully deter neoplastic survival and oncogenesis respectively. Alterations in lipid metabolism are perhaps the least understood cancer. Whereas much is known about the role of DNL, very little is known about the other arm of lipid metabolism: lipolysis. These unknowns are further compounded when considering knowledge in HCC. Once a disease of underdeveloped nations related to HCV, it is now a rapidly growing concern in developed countries as it

has strong ties to the existing obesity and diabetes epidemics. Future considerations should be given to such unknowns in oncogenic metabolism such as the role of lipolysis and lipid catabolism. The work herein seeks to provide address the role ATGL, diet, and lifestyle play in hepatic oncogenesis relevant to the etiologies prevalent in Western societies. We utilized both adenovirus and siRNA-mediated approaches to manipulate ATGL and cyclin D1 both *in-vitro* and *in-vivo* systems and employed exercise and caloric restriction protocols with varying dietary lipid content during long-term carcinogenesis studies in mice. Our studies seek to answer the following questions: Does ATGL-mediated lipolysis attenuate hepatic cell proliferation, a risk factor and hallmark of hepatic oncogenesis? Do lifestyle factors such as regular endurance exercise or caloric restriction influence carcinogen-induced liver cancer? What are potential pathways and molecular targets of interest in hepatic oncogenesis relevant to developed countries?

CHAPTER 2

Cyclin D1 Regulates Adipose Triglyceride Lipase to Influence Mouse and Rat Hepatic Lipid Droplet Metabolism and Cell Proliferation

Jonathan M Ploeger, Sushama Kamarajugadda, Heng Wu, Mara T Mashek, Douglas G Mashek, Jeffrey H Albrecht

Jonathan Ploeger wrote this chapter in its entirety

Obesity is well-documented to promote the development of nonalcoholic fatty liver disease including its more advanced stages such as non-alcoholic steatohepatitis, cirrhosis and hepatocellular carcinoma. Cyclin D1 (D1) is a cell cycle protein that regulates G1/S transition, the rate-limiting step in cell division. Thus, D1 is highly expressed in proliferating hepatocytes and liver cancer and its overexpression (OE) leads to lipid droplet (LD) accumulation in hepatocytes. Conversely, liver-specific knockdown (KD) of adipose triglyceride lipase (ATGL), the rate-limiting enzyme in triacylglyceride (TAG) catabolism, promotes LD accumulation. To characterize a potential link between D1 and ATGL we employed studies in primary hepatocytes and mouse immortalized AML12 cells. As expected, in the absence of mitotic stimuli, D1 OE was sufficient to drive DNA synthesis in primary hepatocytes. However, these effects are abrogated with the OE of ATGL. Knockdown of D1 in the presence of mitogens inhibited DNA synthesis and increased ATGL mRNA expression, but KD or chemical inhibition of ATGL recovered DNA synthesis. Moreover, cell cycle analysis using flow cytometry confirmed that D1 KD increased accumulation of cells in the G0/G1 phase and reduced cells in the S and G2/M phase; these results were reversed with ATGL KD or chemical inhibition. ***Conclusion*****:** These data illustrate that cyclin D1 regulates ATGL to alter hepatic LD metabolism and proliferative capacity in primary hepatocytes and AML12 cells.

Introduction

Hepatocytes possess the ability to rapidly proliferate, but remain quiescent under normal healthy conditions. However, in response to cellular injuries, such as those attributed to chronic liver disease, rapid proliferation of hepatocytes is an important event in restoring homeostasis of the liver (208). In addition to injury response, hepatocyte proliferation may be stimulated by the presence of growth factors and nutrients (209, 210). Identifying the molecular processes that contribute to the latter has become increasingly important with the rising prevalence of obesity-driven liver pathologies in developed countries, including hepatocellular carcinoma (HCC).

Obesity is an established risk factor for non-alcoholic fatty liver disease (NAFLD). Hepatic steatosis, which commonly accompanies obesity and defines NAFLD, can lead to a sequela of events known to cause hepatocellular injury and increase the risk for HCC development (211). This increased risk is in part, due to the induction of molecular mechanisms that initiate hepatocellular proliferation. Insulin resistance induced from excessive caloric intake leads to increased activation of well-established regulators of oncogenic metabolism such as the mechanistic target of rapamycin (mTOR). D1, a downstream target of mTOR (188), is a cell cycle protein that, when bound to cyclin dependent kinase 4, catalyzes the transition from G1 to S phase of the cell cycle. This transition, known as the regulation point or R-point, is an important step in the cell cycle, as advancement past this point commits cells to completion of the proliferative cycle. D1 is highly expressed in the latter stages of NAFLD progression as well

as in HCC (212). In addition to its regulation of a key stage of the cell cycle, D1 has also regulates glycolytic metabolism (198) and *de novo* lipogenesis (213), known metabolic perturbations in advanced liver disease.

In the fed state, insulin leads to phosphorylation of several hepatic proteins including FOXO1 (214). This suppression of FOXO1 results in suppression of gluconeogenesis as well as lipolysis, promoting glycolytic flux and lipogenesis (215–217). Additionally, insulin also promotes Akt phosphorylation of PGC-1α, leading to inhibition of fatty acid oxidation (218); fatty acid oxidation is also inhibited due to insulin's suppressive effect on FOXO1 (219). Conversely, during periods of increased energetic demand such as those experienced during fasting or exercise FoxO proteins promote hepatic TAG catabolism by stimulating the expression of adipose TAG lipase (ATGL) (220) and subsequent lipases (221). Initially identified in adipose tissue (222), ATGL has subsequently been shown to play an important role in other oxidative tissues, including the liver, in promoting TAG catabolism. Our lab has shown that ATGL preferentially partitions hydrolyzed fatty acids (FAs) towards β-oxidation to meet cellular energetic needs (109, 223). Successive studies have further elucidated that in addition to its lipolytic action and FA partitioning, ATGL serves as a key signaling node in fasting metabolism, leading to PPARα/PGC-1α driven β-oxidation through its promotion of the histone deacetylase SIRT1 (224).

While metabolic derangements have been well-characterized as drivers of oncogenesis, little attention has been given to the role of lipid catabolism beyond its role in cachexia (225, 226). Importantly, growth factor signaling

through the mTOR pathway has been shown to inhibit ATGL (127) while catecholamine driven lipolysis represses mTOR signaling in adipocytes (227). Moreover, Akt suppression of FOXO1 is necessary for liver regeneration (228), collectively suggesting a potential link between ATGL and oncogenic signaling. In the present study, we explored the relationship between ATGL and D1, its effect on hepatic lipid metabolism, and implications on hepatocellular proliferation. Results demonstrate that ATGL is a major regulator of hepatocyte proliferation.

Materials & Methods

Animals

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. All experiments were conducted in 10-12-week-old C57BL/6J mice or Wistar rats purchased from Jackson laboratories (Jackson Laboratory, USA). All animals were housed under constant temperature and humidity under a 12/12 light/dark cycle. Animals were fed standard pellet diet *ad libitum* with free access to water.

Surgical Procedures

All partial hepatectomy (PH) surgeries were performed under isoflurane (Sigma-Aldrich, St. Louis, MO) anesthesia. Twenty-four hours prior to PH, animals were subjected to tail vein injection of control or ATGL adenovirus to induce liver specific OE of ATGL. Animals were subjected to sham or 2/3 PH as previously described.[1] Animals were sacrificed at varying time points up to 42-hours post-surgery.

Hepatocyte Isolation and Culture

Mouse and rat primary hepatocytes were isolated by the collagenase perfusion method from 10- to 12-week-old animals. Hepatocytes were isolated as we have described previously.[2] Post isolation, primary hepatocytes were then cultured in William's E media (10% penicillin/streptomycin) with or devoid of insulin (20 mU/ml) and epidermal growth factor (EGF, 10 ng/ml). Four hours after plating,

fresh media was added and cells were transfected with siRNA or transduced with adenovirus targeting cyclin D1, ATGL, and control vectors. Media was subsequently replaced every 24 hours during experiments.

Histology

A medial section of liver tissue was excised and fixed in 10% buffered formalin and subsequently embedded in paraffin blocks. Paraffin-embedded sections were prepared for histopathological and immunohistochemical examinations as previously described (109).

Immunofluorescence

Cells were grown for 72 hours on coverslips. For LD staining, cells were incubated with Bodipy™ Deep Red Neutral Lipid Stain (1 μM; Invitrogen) overnight at 37°C and subsequently fixed with 4% paraformaldehyde for 30 minutes and blocked with 1% BSA in media containing or void of EGF and insulin. Post fixation, nuclei were stained with DAPI (4′, 6-diamidino-2-phenylindole) for 10 minutes followed by mounting onto slides for visualization. All images were acquired with a Nikon A1 Spectral Confocal Microscope (Nikon Ti2000E inverted fluorescence microscope with DIC optics) with a 60X oil objective and 0.6 numerical aperture, and prepared using ImageJ (NIH). Images from 5 different fields per well were captured and experiments were performed in triplicate.

Pulse-Chase Studies

Experiments measuring lipid incorporation (pulse period) to measure TAG turnover or FA oxidation were performed in Williams E media (Invitrogen). Seventy-two hours after plating, cells were pulsed with 500 μM oleate and trace [1-^{14}C] oleate (for turnover) or [1-^{14}C] acetate (for incorporation) for 2 hours. For turnover studies, a subset of cells was harvested at the end of the pulse period to measure radiolabel incorporation into cellular lipid fractions. Remaining cells were washed with PBS and fresh media lacking insulin and serum was added for an additional 6 hours (chase period) followed by collection of media and cells for lipid extraction. To account for differential rates of incorporation, a serial dilution of a TAG standard was prepared and pulse TAG concentrations were normalized to this standard. FAs oxidized during the chase period are expressed as a percentage of the pulse [^{14}C] TAG. Lipids were extracted and separated into different fractions by thin layer chromatography and analyzed as described previously (109). For incorporation studies, cells were pulsed with radiolabeled acetate and treated as described for pulse conditions. Lipids were extracted and analyzed as previously described (109).

Cell Proliferation

DNA synthesis was measured using an ELISA BrdU labeling kit (Roche Diagnostics, Indianapolis, IN) following manufacturer's protocol.

Cell Cycle

Primary rat hepatocytes and AML12 cells were suspended in PBS. Aggregates of cells were gently vortexed to obtain a mono-dispersed cell suspension, with minimal cell aggregation. Cells were fixed by transferring this suspension into centrifuge tubes containing 4.5 mL of 70% ethanol on ice and kept for 2 hours at 4°C. Cells were then centrifuged for 5 min at 300xg. Cell pellet was then suspended in 1 mL of propidium iodide staining solution at 37°C for 10 minutes. The sample was then fed to the flow cytometer and fluorescence of PI bound to DNA at 536 nm excitation, and emission at 617 nm was measured.

mRNA and Protein Analyses

RT-PCR. Cells were lysed and homogenized in Trizol (Thermo Fisher, Waltham, MA). RNA was isolated according to manufacturer's instructions. Five µg of RNA was then subjected to DNAse treatment (Applied Biosystems, Foster City, CA) and cDNA synthesis (Applied Biosystems, Foster City, CA) using random hexamers. cDNA was subjected to RT-PCR using the LC480 SYBR Green kit and the LC 480 Light Cycler (Roche, Indianapolis, IN). Experimental crossing points were normalized to Gapdh (mouse) or Rpl32 (rat). The primer sequences of gene targets has previously been reported (224).

Western Blot. Cells or liver tissue were lysed using Lysis Buffer B (0.1% Triton X-100, 150mM NaCl, 10mM Tris-HCl) and sonicated on ice after incubation on ice for 10 minutes. Cell lysates were spun at 21,000 X g for 10 minutes at 4° C. Supernatant was collected and protein concentration was determined using the BCA method (Thermo Fisher, Waltham, MA). Twenty µg of protein lysate were

boiled with SDS sample buffer and subjected to PAGE followed by overnight transfer to PVDF membrane (Amersham GE Healthcare). Protein electrophoresis was done on poly-acrylamide gels and analyses were performed as described previously (224). Anti-Cyclin D1 antibody (Catalog #04-221) was purchased from Millipore, (Billerica, MA), anti-CDK1 antibody (Catalog# SC-54) was purchased from Santa Cruz (Dallas, TX), anti-ATGL antibody (Catalog# 2183) was purchased from Cell Signaling (Boston, MA). All protein was normalized to Actin (Catalog# NB600-501) and was purchased from Novus (Littleton, CO). The secondary antibody anti-rabbit (Catalog # 7050) conjugated with alkaline phosphatase were purchased from Cell Signaling (Boston, MA).

Statistics

Results are expressed as mean ± standard error of the mean. Statistical analysis was performed using unpaired Student t-test and ANOVA where appropriate. Values of $P<0.05$ were considered statistically significant.

Results

Cyclin D1 and ATGL serves as a mediators of growth factors on LD metabolism and morphology

To determine if modulating D1 and ATGL impacts LD morphology and turnover and any potential connection between the two, we incubated primary hepatocytes with siRNA targeting D1 (siD1) and ATGL (siATGL) for 72 hours. We conducted knockdown studies in the presence of epidermal growth factor

(EGF) and insulin to distinguish between the effects of mitogens and D1 as well as ATGL on LD metabolism and morphology. As expected, siATGL led to significant increases in the expression of *D1*, *CDK1*, and *MCM5* and significant reductions in carnitine palmitoyltransferase 1α (CPT1α) - a marker of FA oxidation (fig. 1A). As expected, hepatocytes treated with ^{12}C bodipy demonstrated significantly increased LD size (fig. 1B).

In contrast to the effects of siATGL, siD1 led to suppression of early phase cell cycle targets including cyclin dependent kinase 1 (*CDK1*), proliferative marker minichromosome maintenance complex component 5 (*MCM5*), and an increase ATGL expression (fig. 1A). Although KD of D1 resulted in significantly more LDs per cell, it also reduced LD size, a phenomenon previously described of cells experiencing conditions conducive of lipid catabolism (229) (fig. 1B).

Because of the morphological changes resultant from D1 closely resembled the effects expected from lipolysis, despite the presence of growth factors, we next determined the effects of D1 KD on hepatocyte TAG metabolism. To do so, we conducted a series of pulse-chase experiments using [1-^{14}C] acetate for assessment of *de novo* lipogenesis and [1-^{14}C] oleate to assess TAG turnover and oxidation in primary rat hepatocytes treated with siRNA's targeting D1. Knockdown of D1 resulted in a significant reduction in [^{14}C] acetate incorporation into hepatocytes (fig. 1C), whereas siD1 resulted in significantly increased TAG catabolism and incomplete fatty acid (FA) oxidation (fig. 1 D & E). Interestingly, in addition to eliciting opposing effects on LD morphology as well as on proliferative and oxidative gene expression, D1 and ATGL knockdown led to significant,

reciprocating elevations in gene expression (fig. 1A). Collectively, these data suggest D1 and ATGL reciprocally regulate hepatic LD metabolism and morphology.

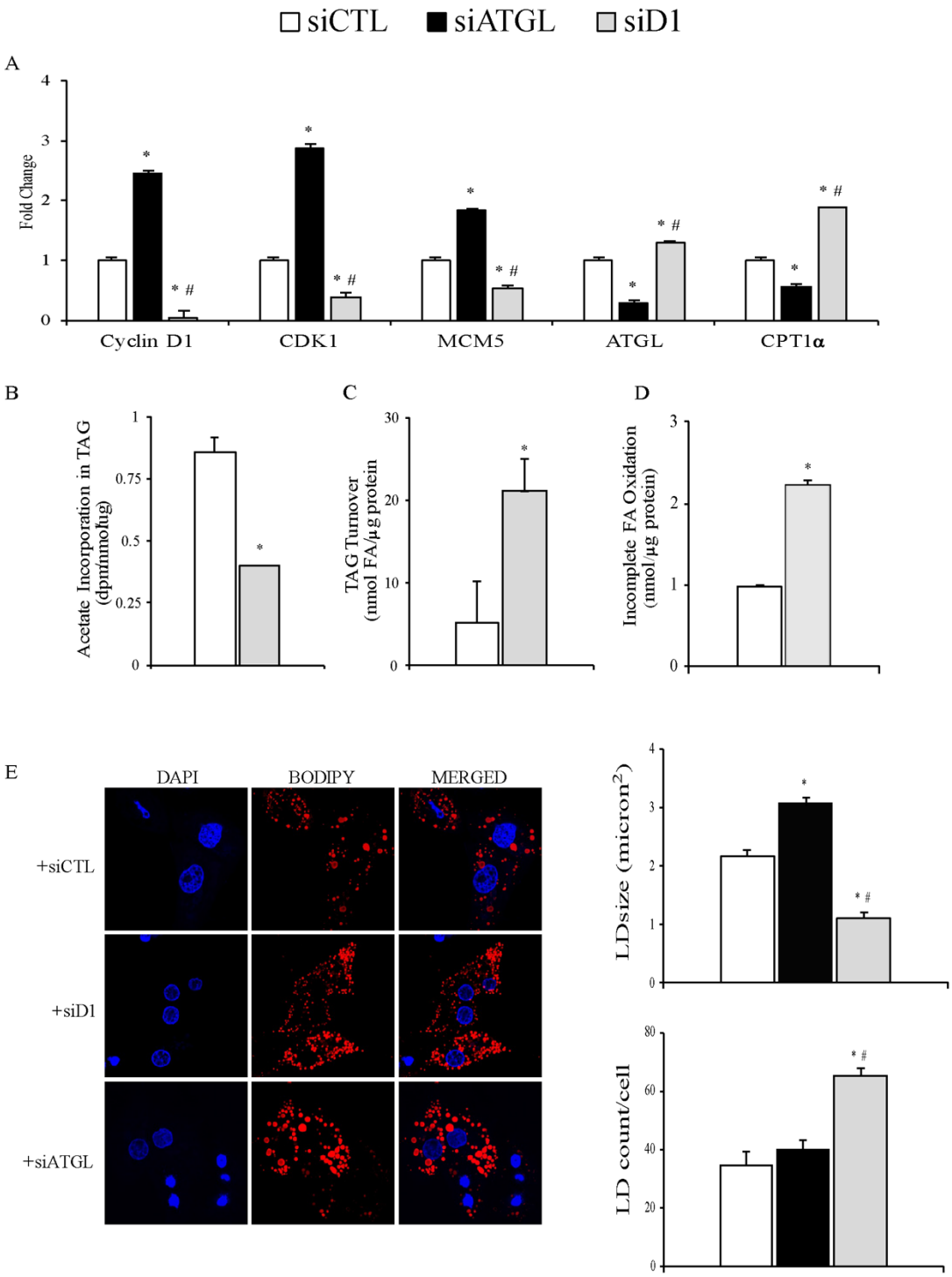

siCTL
siATGL
siD1
A
Fold Change
Cyclin D1
CDK1
MCM5
ATGL
CPT1α
B
Acetate Incorporation in TAG (dpm/mol/ug)
C
TAG Turnover (nmol FA/µg protein
D
Incomplete FA Oxidation (nmol/µg protein)
E
DAPI
BODIPY
MERGED
+siCTL
+siD1
+siATGL
LDsize (micron2)
LD count/cell

Figure. 1. Cyclin D1 knockdown increases LD turnover.

Primary hepatocytes were isolated from Wistar rats and subsequently treated with siRNA targeting cyclin D1 and ATGL. Seventy-two hours after transfection cells were harvested for analysis. (A) mRNA expression of proliferative genes D1, MCM5, and CDK1 as well as oxidative genes ATGL and CPT1α. (B) Inhibition of cyclin D1 lowered [1-^{14}C] acetate incorporation into primary hepatocytes. Cells pulsed with [1-^{14}C] oleate had media changed after 6 hours to measure (C) TAG turnover and fatty acid oxidation (D). Primary hepatocytes were incubated with C^{12} bodipy to stain for neutral lipids overnight and DAPI for nuclei and LD size and number was determined using ImageJ. *$P<0.05$ compared to siCTL, $^{\#}P<0.05$ compared to siATGL.

Overexpression of D1 alters LD metabolism and morphology independently of mitogens

Because KD of D1 and ATGL in primary rat hepatocytes elicited opposing effects on gene expression, LD metabolism, and morphology in the presence of growth factors, we next analyzed these effects independent of mitogens through overexpression studies using adenoviruses. Where OE of D1 significantly increased expression of *D1* and *CDK1* while repressing *ATGL*, OE of ATGL significantly repressed expression of *D1* and *CDK1* (fig. 2A & B). [^{14}C] acetate incorporation into hepatocellular LDs, a measure of *de novo* lipogenesis, was significantly increased with D1 OE (fig. 2C). TAG turnover and incomplete oxidation of fatty acids were significantly reduced following D1 OE (fig. 2D-E). Finally, D1 OE significantly increased both size and number of LD's independent of EGF and insulin, whereas ATGL OE significantly reduced LD size despite mitogen stimulation (fig. 1F). Collectively, these data demonstrate D1 alters LD metabolism, in opposition to ATGL, in part through the repression of catabolic lipid metabolism.

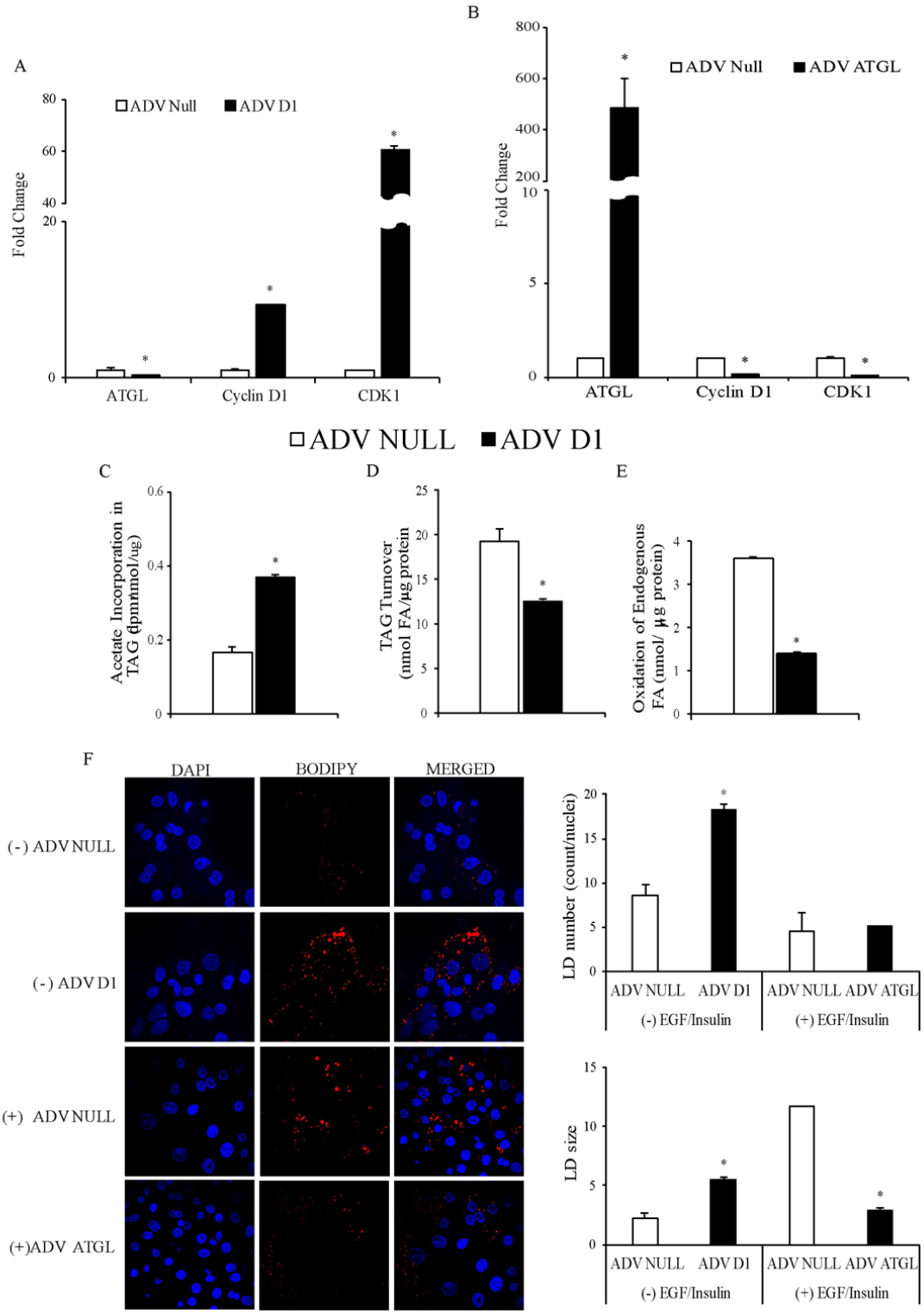

A
ADV Null
ADV D1
Fold Change
ATGL
Cyclin D1
CDK1
B
ADV Null
ADV ATGL
Fold Change
ATGL
Cyclin D1
CDK1
ADV NULL
ADV D1
C
Acetate Incorporation in TAG (dpm/nmol/ug)
D
TAG Turnover (nmol FA/µg protein
E
Oxidation of Endogenous FA (nmol/ µg protein)
F
DAPI
BODIPY
MERGED
(-) ADV NULL
(-) ADV D1
(+) ADV NULL
(+)ADV ATGL
LD number (count/nuclei)
ADV NULL
ADV D1
(-) EGF/Insulin
ADV NULL
ADV ATGL
(+) EGF/Insulin
LD size
ADV NULL
ADV D1
(-) EGF/Insulin
ADV NULL
ADV ATGL
(+) EGF/Insulin

Figure. 2. Cyclin D1 overexpression represses ATGL expression and LD turnover.

Primary hepatocytes were isolated from Wistar rats and subsequently treated with adenoviruses targeting cyclin D1 and ATGL. Seventy-two hours after transfection cells were harvested for analysis. (A & B) mRNA expression of proliferative genes targets D1 and CDK1 as well as ATGL. (C) Inhibition of cyclin D1 lowered [1-^{14}C] acetate incorporation into primary hepatocytes. Cells pulsed with [1-^{14}C] oleate had media changed after 6 hours to measure (D) TAG turnover and fatty acid oxidation (E). (F) Primary hepatocytes were incubated with C^{12} bodipy to stain for neutral lipids overnight and DAPI for nuclei and LD size and number was determined using ImageJ. *$P<0.05$ compared to siCTL, $^{\#}P<0.05$ compared to siATGL.

ATGL attenuates D1 mediated hepatocyte proliferation

Cyclin D1 is an important mediator of hepatocellular proliferation. Therefore, because manipulation of D1 led to opposing effects on ATGL mRNA expression, which extended to lipid metabolism, we next sought to determine whether metabolic results were recapitulated in hepatocellular proliferation. As expected, D1 OE in primary rat hepatocytes cultured in media devoid of EGF and insulin was sufficient to increase DNA synthesis (fig. 3A). Alternatively, hepatocytes overexpression of ATGL attenuated the effects of EGF and insulin on DNA synthesis (fig. 3B). D1 knockdown led to reduced DNA synthesis, but simultaneously knocking down ATGL attenuated these effects (fig. 3C). In addition to siRNA treatments, primary hepatocytes cultured with the ATGL specific inhibitor ASTAT had increased DNA synthesis compared to vehicle controls (fig. 3D). To confirm that the alterations in DNA synthesis were due to effects on D1, we analyzed primary hepatocytes exposed to siRNA by flow cytometry. As expected, D1 KD led to aggregation of cells in the G0/G1 phase and a reduction in cells transitioning into the S and G2/M phase (fig. 3E). Additionally, ATGL KD led to an increase in cells progressing into the S and G2/M phase, while simultaneous D1 and ATGL KD led to a modulation of cell cycle progression in between individual siRNA treatments (fig. 3E). Collectively, these data support ATGL as a deterrent of hepatocyte proliferation in a D1 dependent manner.

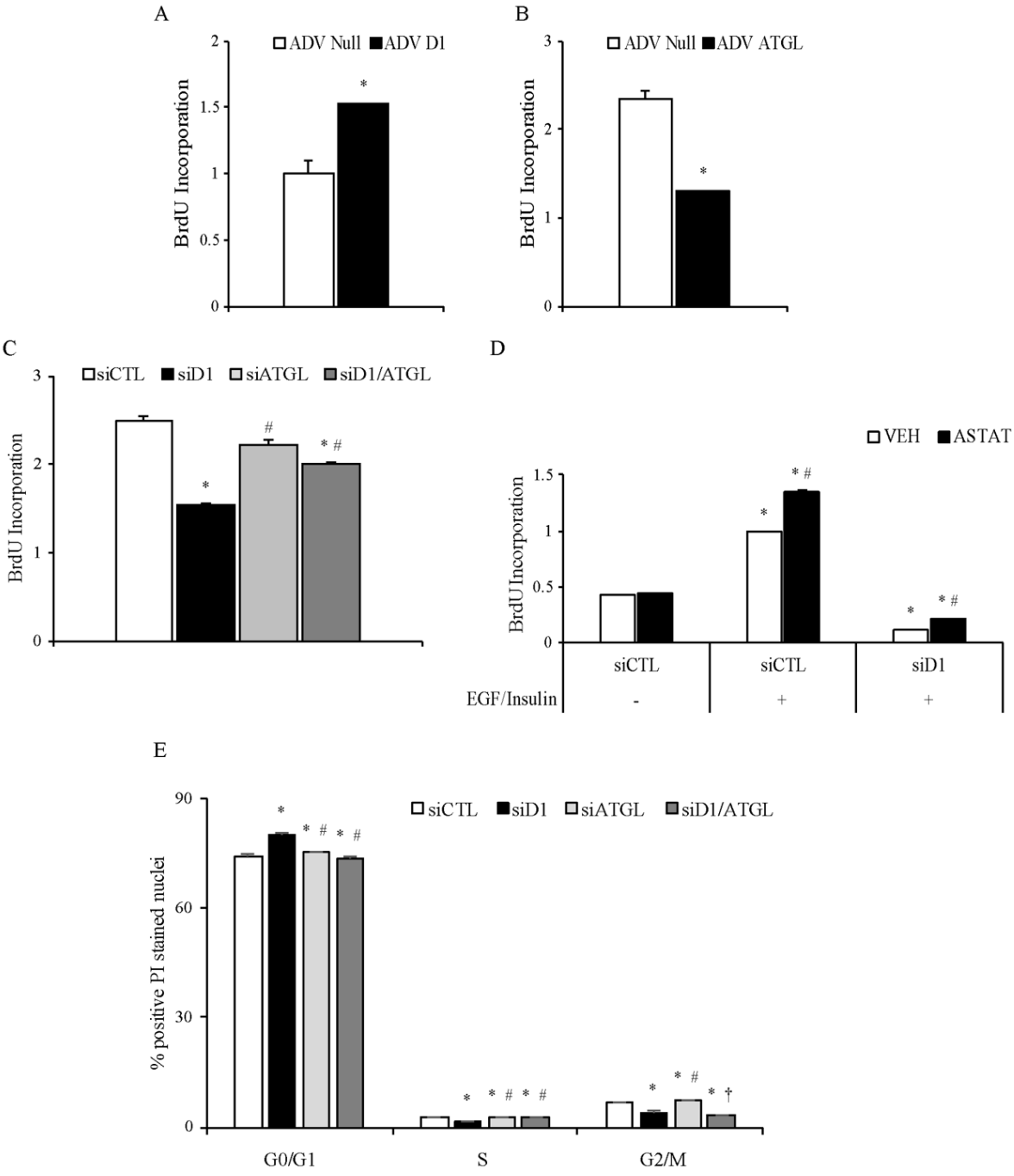

A
ADV Null
ADV D1
BrdU Incorporation
B
ADV Null
ADV ATGL
BrdU Incorporation
C
siCTL
siD1
siATGL
siD1/ATGL
BrdU Incorporation
D
VEH
ASTAT
BrdU Incorporation
siCTL
siCTL
siD1
EGF/Insulin
E
siCTL
siD1
siATGL
siD1/ATGL
% positive PI stained nuclei
G0/G1
S
G2/M

Figure. 3. ATGL and Cyclin D1 differentially regulate proliferation in primary hepatocytes.

Primary hepatocytes were isolated from Wistar rats and subsequently treated with adenoviruses targeting cyclin D1 and ATGL. Seventy-two hours after transfection cells were harvested for analysis. (A) Cyclin D1 increased incorporation of BrdU whereas (B) ATGL inhibits BrdU incorporation despite the presence of growth factors. (C) ATGL and cyclin D1 differentially regulate DNA synthesis. (D) Cells treated with ATGL specific inhibitor ASTAT show increased DNA synthesis both in the presence of growth factors and with D1 knockdown. (E) Primary hepatocytes were stained with propidium iodide and analyzed for stage of the cell cycle using flow cytometry. *$P<0.05$ compared to siCTL, $^{\#}P<0.05$ compared to siD1, $^{\dagger}P<0.05$ compared to siATGL.

Hepatocyte effects on gene expression, LD metabolism, and proliferation are recapitulated in AML12 cells

To recapitulate findings from primary rat hepatocyte experiments, we next investigated the effects of ATGL and D1 KD in the immortalized hepatocyte cell line AML12. As before, D1 and ATGL knockdowns reciprocally suppressed the expression of one another (fig. 4A). Additionally, treatment with siRNA targeting D1 alone, or in combination with ATGL, led to a reduction in total lipid area quantified from oil red o staining, whereas siATGL led to a significant increase in LD area (fig. 4B). Pulse-chase studies confirmed these observations showing that siD1 led to an increase in TAG turnover and incomplete oxidation of hydrolyzed FAs (fig. 4C & D). DNA synthesis was increased with siATGL, but was reduced with siD1 or siD1/ATGL treatments (fig. 4E). Inhibition of ATGL activity with ASTAT led to a significant increase in DNA synthesis despite the absence of EGF and insulin (fig. 4F). Similar to the data generated in hepatocytes, D1 KD increased the number of AML12 cells in the G0/G1 phase leading to a reduction of cells progressing through subsequent phases of the cell cycle (fig. 4G). Conversely, siATGL led to a significant increase in cells progressing to the S phase of the cell cycle and the combined KD of D1 and ATGL led to cells fluxing through the cell cycle in between the individual treatments (fig. 4G). Collectively, these data demonstrate the antagonistic cross-talk between ATGL and D1 extends beyond isolated primary hepatocytes.

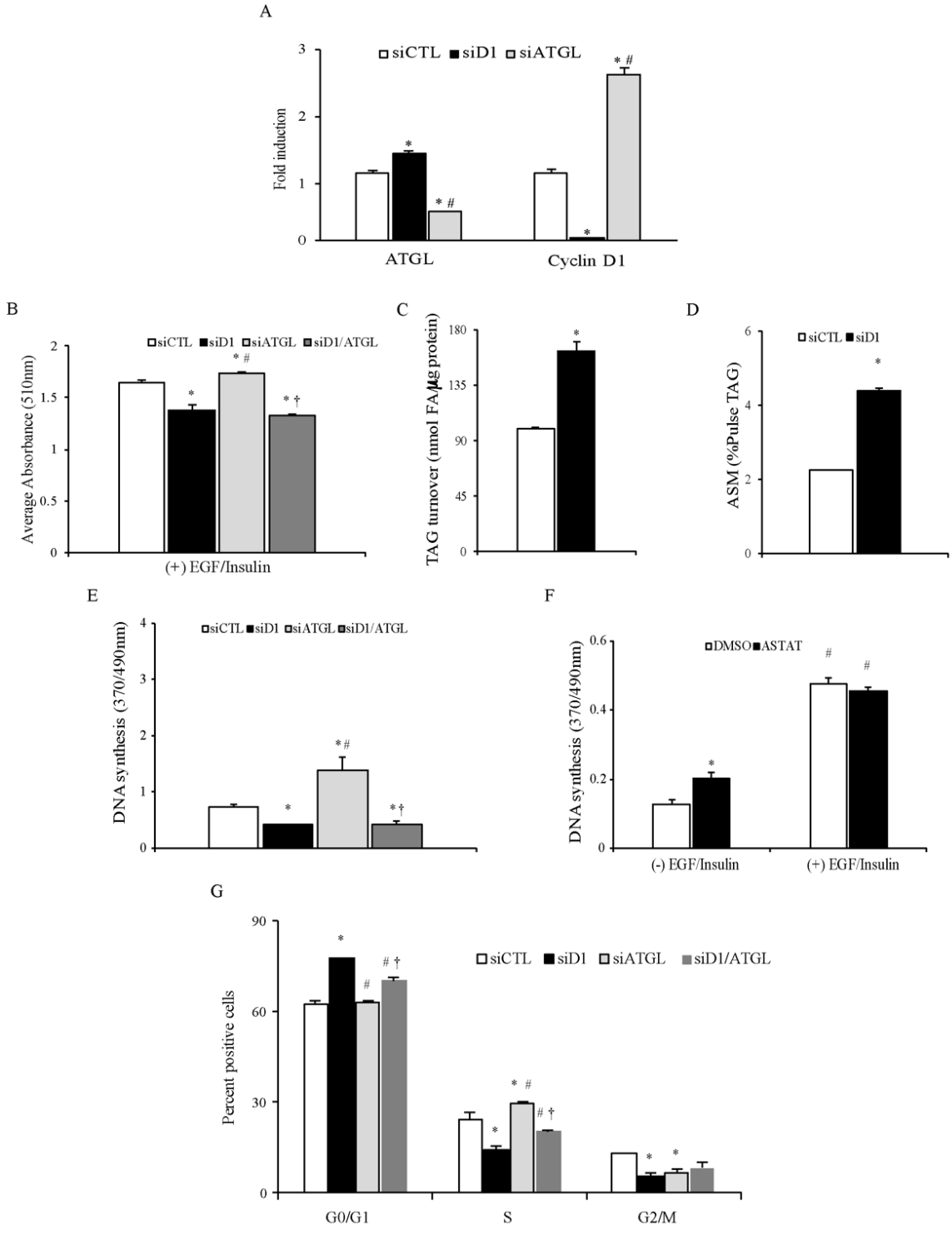

A
siCTL siD1 siATGL
Fold induction
ATGL
Cyclin D1
B
siCTL siD1 siATGL siD1/ATGL
Average Absorbance (510nm)
(+) EGF/Insulin
C
TAG turnover (nmol FA/μg protein)
D
siCTL siD1
ASM (%Pulse TAG)
E
siCTL siD1 siATGL siD1/ATGL
DNA synthesis (370/490nm)
F
DMSO ASTAT
DNA synthesis (370/490nm)
(-) EGF/Insulin
(+) EGF/Insulin
G
siCTL siD1 siATGL siD1/ATGL
Percent positive cells
G0/G1
S
G2/M

Figure. 4. ATGL and Cyclin D1 differentially regulate proliferation in AML12 cells.

AML12 cells were treated with siRNA targeting cyclin D1 and ATGL or the ASTAT and cultured for 72 hours. (A) mRNA expression of D1 and ATGL. (B) Cells stained with oil-red-o had total lipid determined using ImageJ. (C) ATGL inhibits BrdU incorporation despite the presence of growth factors. Cells pulsed with [1-^{14}C] oleate had media changed after 6 hours to measure TAG turnover (C) and fatty acid oxidation (D). DNA synthesis measured using BrdU incorporation in cells treated with siRNA (E) and ASTAT (F). (G) AML12 cells were stained with propidium iodide and analyzed for stage of the cell cycle using flow cytometry. *$P<0.05$ compared to siCTL, $^{\#}P<0.05$ compared to siD1, $^{\dagger}P<0.05$ compared to siATGL.

ATGL OE limits liver regeneration

To investigate the role of ATGL and D1 in an *in vivo* system, we performed a 70 percent partial hepatectomy, which is a common model for investigating liver regeneration and proliferation (230). Liver tissue harvested 42 hours post HR exhibited a significant reduction in staining of the proliferative marker Ki-67 and a marked reduction in steatosis in response to ATGL OE (fig. 5A). Western blots demonstrated a significant increase in D1 and CDK1 with GFP treatment compared to sham surgery animals, whereas ATGL OE reduced these markers compared (fig. 5B). This data collectively points to an antagonistic cross-talk between ATGL and D1, which results in alterations in LD biology, lipid metabolism, and reductions in hepatocellular proliferation in an *in vivo* model.

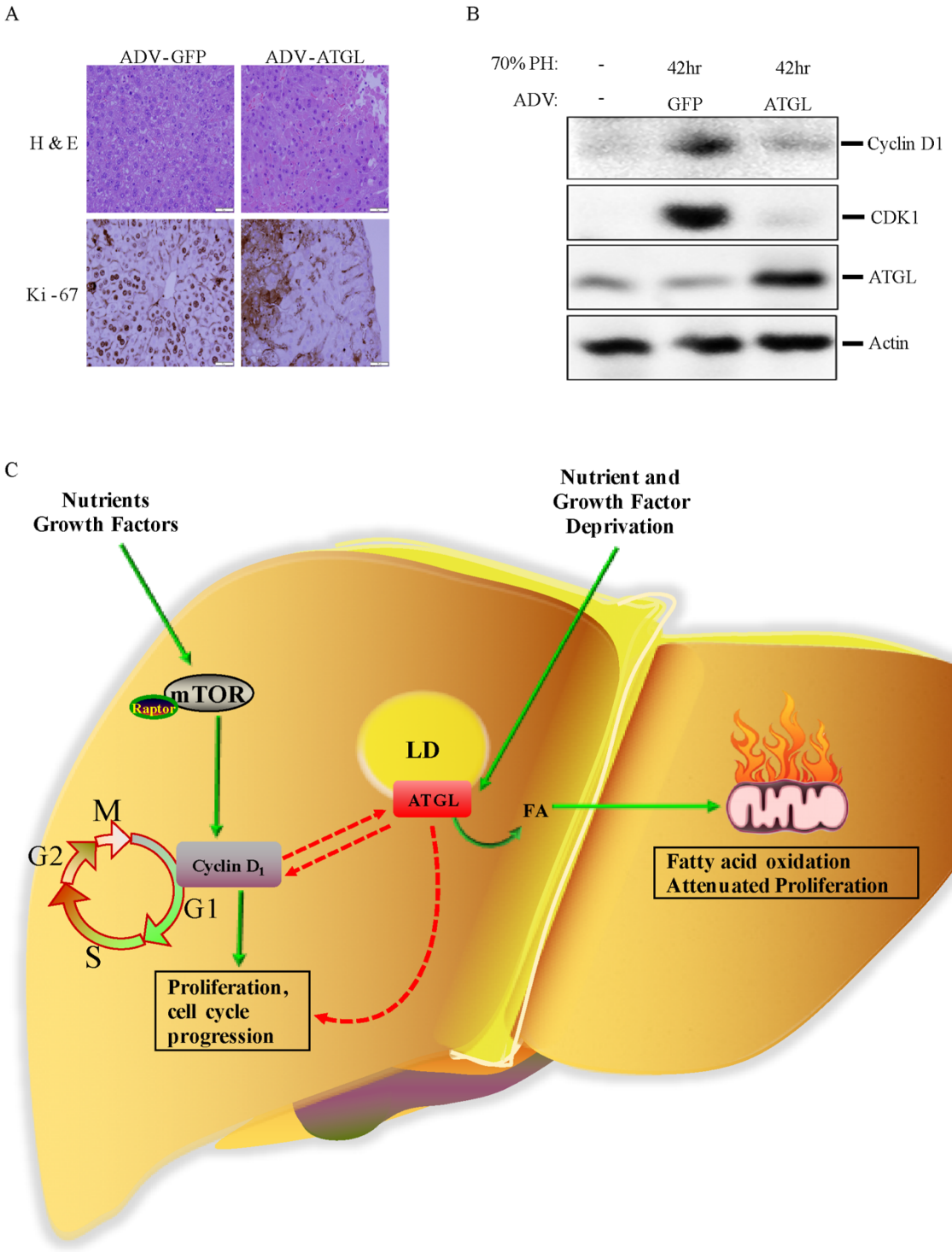

A
ADV-GFP
ADV-ATGL
H & E
Ki-67
B
70% PH:
ADV:
42hr
42hr
GFP
ATGL
Cyclin D1
CDK1
ATGL
Actin
C
Nutrients
Growth Factors
Nutrient and
Growth Factor
Deprivation
mTOR
Raptor
LD
ATGL
FA
M
G2
G1
S
Cyclin D1
Fatty acid oxidation
Attenuated Proliferation
Proliferation,
cell cycle
progression

Figure. 5. ATGL inhibits hepatocyte regeneration after partial hepatectomy.

Forty-two hours after completion of 70 percent partial hepatectomy, animals were sacrificed and liver tissue was analyzed for lipid accumulation (A) and the proliferative marker Ki-67 (B). Western blot of hepatic tissue demonstrated mice treated with ATGL adenovirus had reduced expression of cyclin D1 and CDK1 protein compared to GFP control and sham surgery treated animals. (C) Infographic of summary of findings.

Discussion

Alterations in energy metabolism resulting from overconsumption of calories are well known to increase the risk of obesity and NAFLD. Once thought of as inert organelles, LD's are now widely considered to play a dynamic role in a wide range of cellular processes. Accumulation of LD, the hallmark of NAFLD, has attracted much attention in attempting to explain the biology of this metabolic state and its links to a spectrum of diseases.

The accumulation of hepatic LDs can result from either an increase in synthesis (*de novo lipogenesis or fatty acid uptake*) or a suppression of turnover (lipolysis). Indeed, both mechanisms have demonstrated relevance as it pertains to the development of steatosis (109, 160, 221, 231). In the fed state, the liver increases TAG synthesis due to increased glucose catabolism. This is in part explained by nutrients and growth factors well-documented effects on cell signaling pathways promoting lipogenesis. One key signaling node, mTOR, has been shown to activate cyclin D1 in response to such stimuli (232). Importantly, D1 expression has been shown to influence both glucose metabolism and lipid synthesis (198, 213, 233). In the present study, D1 was both shown to be a mediator of growth factors (fig. 1) and sufficient in the absence of growth factors (fig. 2) to drive hepatocellular LD accumulation and cell proliferation.

Lipolysis, which is initiated during periods of fasting or exercise, provides fatty acids for oxidation to meet cellular energy needs. Our laboratory has previously demonstrated that hepatic ATGL expression is sufficient and necessary in preventing and reversing steatosis (109). Additionally, growth factors suppress

ATGL-driven lipolysis (127) and lipolysis has been shown to antagonize mTOR (227) in adipocytes, suggesting a possible extension of these effects in the liver. Indeed, data from this study recapitulates previous findings demonstrating ATGL as a key enzyme in LD catabolism and identify cross-talk relationship with D1 that determines hepatocellular TAG turnover, FA oxidation, and LD size and number.

Paradoxically, accumulation of lipid leading to steatosis can further lead to subsequent pathologies, yet accumulation of LD is necessary for proliferating cells (160). In the liver, the loss of LD associated protein perilipin 2 is sufficient to ameliorate steatosis and reduce D1 expression, leading to a delay in liver regeneration (160). This is relevant to the current study since perilipin 2 is highly expressed in fatty livers (234) and is known to antagonize lipolysis (235). D1 is also widely recognized as a key signaling node in hepatocellular proliferation (188). Supporting this work, the results here show that in addition to effects on LD morphology and metabolism, D1 promoted DNA synthesis even in the absence of growth factor stimulus. The data also show for the first time that ATGL expression can antagonize the effects of growth factors in driving cell proliferation and rescue the stimulatory effects of D1. Furthermore, results from experiments inhibiting lipolysis with ASTAT indicate that the activity of ATGL and not merely its expression play an important role in mitigating the effects of D1 on LD metabolism and hepatocyte proliferation. Finally, results from the present study reveal that ATGL's inhibitory effects on proliferation of hepatocytes and immortalized liver cells are carried out in a D1 dependent manner, limiting

transition from the G1 into the S phase; importantly, the inhibitory effects of ATGL on proliferation were recapitulated in an *in vivo* model suggesting translational applications.

In conclusion, data from this work shows for the first time that cyclin D1 increases lipid accumulation in hepatocytes and mouse livers, in part, through its suppression of ATGL catalyzed lipolysis. Furthermore, the work also points to a cross-talk relationship between the two targets and further suggests ATGL activity is sufficient and necessary in limiting both metabolic and proliferative derangements associated with obesity and risk for subsequent pathologies. Future work will need to focus on identifying the mechanism explaining these results, which will provide further insight into metabolic and pathologic features of obesity related diseases, potentially opening the door for translational investigations in human populations.

Chapter 3

Caloric Restriction Prevents Carcinogen-Initiated Tumorigenesis Cancer

Jonathan M. Ploeger, Juan C. Manivel, Lauren N. Boatner, Douglas G. Mashek

This work is planned to be published in Cancer Prevention Research

Jonathan Ploeger wrote this chapter in its entirety

Caloric restriction (CR) and endurance exercise elicit wide-ranging health benefits including reduced risk of select cancers. In addition, diet composition influences oncogenesis, although its interactions with exercise and CR are not well understood. Therefore, to investigate the potential interactions between diet and lifestyle interventions on liver tumorigenesis, the carcinogen diethylnitrosamine (DEN) was administered to 72 male C57Bl/6 mice that were subsequently fed diets enriched with lard (CTL) or olive oil (OO) and were further stratified to voluntary wheel running (Ex) or 30% CR for 49 weeks. While Ex and diet composition did not influence liver oncogenesis, CR prevented hepatic tumor formation. Additionally, CR reduced steatosis, hepatocyte ballooning, inflammation and immune cell infiltration, all of which are hallmarks in the progression of non-alcoholic fatty liver disease (NAFLD) to liver tumorigenesis. RNA sequencing of non-transformed liver tissues revealed changes in metabolic pathways and reduced inflammation, cytokine production, stellate cell activation and migration, and genes associated with liver injury and oncogenesis. These data demonstrate that CR protects against steatosis, liver inflammation, and liver injury and is a robust deterrent of carcinogen-induced hepatic oncogenesis.

Introduction

Alterations in energy metabolism resulting from overconsumption of calories are well known to increase the risk of obesity, which accounts for nearly 20% of all cancers in the U.S. (27). In addition, prospective epidemiological studies have shown that obesity increases the risk of mortality from nearly every major form of cancer including liver cancer (29). The mortality rate for NAFLD-derived hepatocellular carcinoma (HCC), the most common liver cancer, is higher than other etiologic factors such as hepatitis viral infection with mortality rates as high as 61% within 1 year of diagnosis (236). While epidemiologic studies have linked obesity to increased cancer risk and mortality, the molecular mechanisms underlying this relationship have not been fully elucidated.

Adherence to the Mediterranean Diet (MD), considered the gold-standard for diets, is associated with a lower incidence of cancer and has been shown to reverse the effects of NAFLD in humans (237). OO, which is rich in monounsaturated fatty acids (MUFAs), is a food regularly consumed by adherents of the MD has been reported to provide numerous health benefits (238). Studies in mice and humans have shown consumption of high MUFA containing oils, such as olive oil, to be protective against NAFLD development, even with diets high in fat content (239).

Sedentary lifestyles are well-established contributors to obesity, metabolic disease, and tumorigenesis (27). Exercise on the other hand, has been shown to reduce mortality and protecting against numerous lethal disease such as cancer (240). Of the two major forms of exercise, endurance and resistance, the former

has proven more beneficial to cardiovascular health (241) and obesity (242). Endurance exercise is also an effective therapeutic tool in reversing NAFLD, a major risk factor for liver tumorigenesis (243). Given these benefits, it is perhaps not surprising that numerous studies have demonstrated endurance exercise to be protective against liver tumorigenesis (244). Importantly, physical activity by adherents of the MD lowers the risk of morbidity and mortality more than their inactive counterparts (245) suggesting a cooperative role between the MD and lifestyle interventions.

Unlike increased morbidity and mortality associated with sedentary lifestyles, CR has a long-standing history of extending disease free longevity (246). CR has also been shown to prevent or delay mammary (247), prostate (248), brain (249), intestinal (250), and pancreatic cancers (251). Yet, despite CR proven effectiveness as a therapy in reversing and preventing NAFLD (252), no hepatic CR oncogenesis studies have investigated exposures and pathologies relevant to Western societies, such as through NAFLD. Additionally, while CR and exercise lower the risk for numerous cancers, the molecular pathways involved in elucidating these effects are not fully understood. Therefore, to investigate any collaborative link between dietary fat composition, akin to a Western versus a Mediterranean diet, and either regular aerobic exercise or CR, a long-term liver carcinogenesis study using DEN was employed. Results show dietary composition and exercise had no effect on tumor development, but CR reduced liver inflammation and injury and abrogated tumorigenesis.

Materials & Methods

Animals and Chemicals

Four-week-old male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All mice were maintained at the University of Minnesota Animal Facilities in accordance with the Institutional Animal Care Guidelines and all experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Mice were individually housed under a 12:12 light/dark cycle with free access to water. DEN and glyceryl triochtonoate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The final injection mixture was prepared by suspending DEN in glyceryl triochtonoate to a concentration of 2.5 mg/ml just prior to injection. At 4 weeks of age, all mice were given a single intraperitoneal injection of DEN mixture (25 mg/kg body weight) to induce liver tumorigenesis. Upon injection, mice were individually housed in cages containing 1/8" irradiated corncob bedding for the duration of the study. A subset of mice (n=20) were housed in modified cages containing a running wheel, which was retrofitted with a bicycle computer to track weekly distance (Km) and wheel revolutions as a means of tracking activity levels. To achieve CR, daily food consumption was determined by weighing lard and OO diet intakes on consecutive days weekly. Daily aliquots of CR CTL and OO diets were prepared by weighing 70% of the average daily food weight consumed by sedentary (Sed) animals; aliquots were prepared weekly and stored at 4°C. Food consumption and body weight were measured weekly. Forty-nine weeks after injection, animals were sacrificed for tissue and serum collection

after a 4 hour fast. After anesthesia, livers were harvested by surgical resection and surface nodules were counted.

Diets

To examine differences between dietary fat typically consumed by Western and Mediterranean inhabitants regarding tumorigenesis, Sed (n=32) and Ex mice (n=20) were fed purified diets (AIN-93G) purchased from Harlan Teklad (Indianapolis, IN, USA). Sed and Ex mice were stratified to receive diets where 25% of the kcal were derived from fat (15% of Kcal of fat was derived from either lard (TD.150656) or olive oil (TD.150657) and soybean oil contributed the remaining 10%) (Table S1). CTL CR or OO CR diets (TD150658 and TD.150659 respectively) were made to contain 30% less macronutrient content compared to *ad libitum* diets (10/10/10 CHO/FAT/PRO). CR diets were then supplemented with added vitamin and mineral mix (AIN-93G-MX and AIN93-VX respectively) to ensure animals remained nutrient sufficient. If initiated at a young age, CR has been shown to have deleterious developmental effects (253). Therefore, we gradually restricted CR mouse food in 10% increments over three weeks beginning at 5 weeks of age until a 30% reduction was achieved (Fig. S1A).

NAFLD & NASH Scoring

Hepatocellular steatosis, inflammation, Mallory body and Kupffer cell infiltration, and ballooning were scored by a pathologist (J.C.M.) (VA Medical Center, St. Paul, MN) blinded to all treatment groupings.

Immunohistochemistry and Histological Analysis

A medial section of liver tissue was excised and fixed in 10% buffered formalin and subsequently embedded in paraffin blocks or placed in optimal cutting media and slowly frozen in liquid nitrogen before paraffin embedding. Paraffin-embedded sections were prepared for histopathological and immunohistochemical examinations as previously described (109). Cryo blocks were stored at -80°C until preparation. Cryosections of hepatic tissue were stained with Oil-Red-O as previously described (109) and analyzed using ImageJ to determine lipid area per field of 5 randomly selected views per animal from respective treatment groups.

RNA extraction, library preparation, and next-generation sequencing

Six samples of non-transformed, snap frozen hepatic tissue were randomly selected using a random number generator from the CTL Sed and CTL CR groups for RNA-sequencing. RNA was extracted from snap frozen liver tissue using the RNEasy RNA mini kit purchased from Qiagen (Valencia, CA, USA). A total of 12 RNA samples [6 samples per group x 2 groups (CTL & CR)] were sent to University of Minnesota Genomics Core (UMNGC) for quality check, library preparation, and sequencing. Eukaryotic RNA isolates were quantified using a fluorimetric RiboGreen assay and total RNA integrity was assessed using capillary electrophoresis (e.g., Agilent BioAnalyzer 2100). Only samples higher than 1 microgram with a RIN of 8 or greater proceeded to sequencing. Total RNA samples were converted to Illumina sequencing libraries using Illumina's Truseq RNA Sample Preparation Kit (Cat. # RS-122-2001 or RS-122-2002) or stranded

mRNA Sample Preparation kit (Cat. # RS-122-2101). One microgram of total RNA was oligo-dT purified using oligo-dT coated magnetic beads, fragmented and then reverse transcribed into cDNA. The cDNA was fragmented, blunt-ended, and ligated to indexed (barcoded) adaptors and amplified using 15 cycles of PCR. Final library size distribution was validated using capillary electrophoresis and quantified using fluorimetry (PicoGreen) and via Q-PCR. Indexed libraries were then normalized, pooled and size selected to 320bp +/- 5% using Caliper's XT instrument. Truseq libraries were hybridized to a single read flow cell and individual fragments were clonally amplified by bridge amplification on the Illumina cBot. Once complete, the flow cell was loaded on the HiSeq 2500 and sequenced. Upon completion of read 1, an 8bp forward and 8bp reverse (i7 and i5) index read was performed. Base call files for each cycle of sequencing were generated by Illumina Real Time Analysis (RTA) software. Primary analysis and de-multiplexing were performed using Illumina's bcl2fatstq software version 2.17.1.14.

For the RNA sequencing analysis, 50bp FastQ Reads (n=12 Million per sample) were trimmed using Trimmomatic (v 0.33) enabled with the optional "-q" option; 3bp sliding-window trimming from 3' end requiring minimum Q30. Quality control checks on raw sequence data for each sample were performed with FastQC. Read mapping was performed via Bowtie (v2.2.4.0) using the UCSC mouse genome (mm10) as reference. Gene quantification was done via Cuffquant for FPKM values and Feature Counts for raw read counts. Differentially expressed genes were identified using the edgeR (negative

bionomial) feature in CLCGWB (Qiagen, Valencia, CA) using raw read counts. The generated list was filtered based on a minimum 2X Abs Fold Change and Bonferroni corrected $p < 0.05$. These filtered genes were then imported to Ingenuity Pathway Analysis Software (Qiagen, Valencia, CA) for pathway identification.

Ingenuity Pathway Analysis (IPA)

Isoforms that exhibited a log2 fold change greater than 1 and a false detection rate (FDR) less than 0.05 were subjected to Ingenuity Pathway Analysis (IPA 4.0, Ingenuity Systems, www.Ingenuity.com). The input isoforms were mapped to IPA's knowledge bases, and the relevant biological functions, networks, and pathways related to the treatment were identified.

Reactive Oxygen Species

Reactive oxygen species were detected from 50µg of non-transformed hepatic tissue homogenate using the OxiSelect^{TM} in-vitro reactive oxygen species kit (Cell Biolabs, San Diego, CA) per manufacturer's instructions.

Serum Analyses

Serum was isolated from whole blood samples taken via cardiac puncture at the time of animal sacrifice and kept on ice until centrifugation. Samples were spun at 5,000 X g for 10 minutes and supernatant was aliquoted and stored at -80°C. Non-esterified fatty acids (NEFA) and total ketone bodies were analyzed using the free fatty acid and total ketone body kits respectively purchased from Stanbio labs (Boerne TX, USA). Total ketone bodies and serum insulin were analyzed

using the total ketone body isolation and insulin kits respectively purchased from Wako (Richmond, VA).

Statistical Analysis

Statistical analysis was performed using Graphpad Prism7. Data are expressed as mean ± SEM. Statistical analyses were performed using Student *t* test or ANOVA where appropriate. Statistical analysis for RNA-seq is described in detail under the respective methods. Differences were considered significant at $P < 0.05$.

Gene Sequencing Data deposition

RNA-seq data have been uploaded to the NCBI Sequencing Read Archive, www.ncbi.nlm.nigh.gov/sra (accession no. SRP103046). This article contains supporting information online at www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP103046.

Results

Phenotypic effects of diet, Ex and CR

To determine the independent and potentially synergistic effects of exercise, CR, and diet lipid composition on liver cancer, 4-week-old male C57BL/6 mice were given a single intraperitoneal of DEN. Subsequently, mice were stratified to receive diets with fat content being enriched in either lard or OO (Table S1). Mice were further subcategorized to either remain Sed or given unlimited access to running wheels or incremental CR (Fig. S1A). Sed and Ex mice were fed *ad libitum* whereas CR mice were fed 70% of calories of their respective Sed CTLs.

Mice with access to running wheels ran over 45 Km during the first week of exposure, but running wheel use gradually declined especially during the first 6 months (Fig. 1A-B; Fig. S1B). Within the Ex groups, mice fed the OO diet ran significantly more than those fed the CTL diet over the duration of the study (Fig. 1B). However, like total running wheel use, the increased running of mice fed the OO diet was only evident during the first 6 months of the study (Fig. S1B). OO significantly reduced food intake in Sed mice, but not in those in the Ex group (Fig. 1C-D). As expected, CR reduced body weight gain, which was unaffected by dietary lipid composition (Fig. 1E & F). Consistent with reduced body weight, both CR groups had smaller inguinal fat pads (Fig. S1C) although the reduction in epididymal fat pads was only observed in the CR OO mice (Fig. S1D). Over the course of the study, both Ex groups gained more body weight than their Sed CTLs (Fig. 1F) perhaps due to increased muscle mass, which commonly occurs with exercise training (254). There were not treatment effects on food efficiency defined as body weight gain/food intake (Fig. S1E).

Supplementary Table 1. Description of components (g/Kg) and macronutrient content (% kcal) of diets.

	CTL (TD.150656)	OO (TD.150657)	CR CTL (TD.150658)	CR OO (TD.150659)
	Sed and Ex mice		CR mice	
Components	g/Kg			
Casein	200.0	200.0	196.0	196.0
L-Cystine	3.0	3.0	2.94	2.94
Corn Starch	359.186	359.186	349.98	349.98
Maltodextrin	132.0	132.0	129.2	129.2
Sucrose	100.0	100.0	98.0	98.0
Lard	65.0	-----	64.2	-----
Olive Oil	-----	65.0	-----	64.2
Soybean Oil	43.2	43.2	42.7	42.7
Cellulose	50.0	50.0	49	49
Mineral Mix, AN-93G-MX	35.0	35.0	50	50
Vitamin Mix, AN-93-VX	10.0	10.0	14.29	14.29
Choline Bitartrate	2.5	2.5	3.57	3.57
TBHQ, antioxidant	0.014	0.014	0.02	0.02
	% kcal			
Protein	17.9	17.9	17.7	17.7
Carbohydrate	57.1	57.1	57.3	57.3
Fat	25	25	25	25

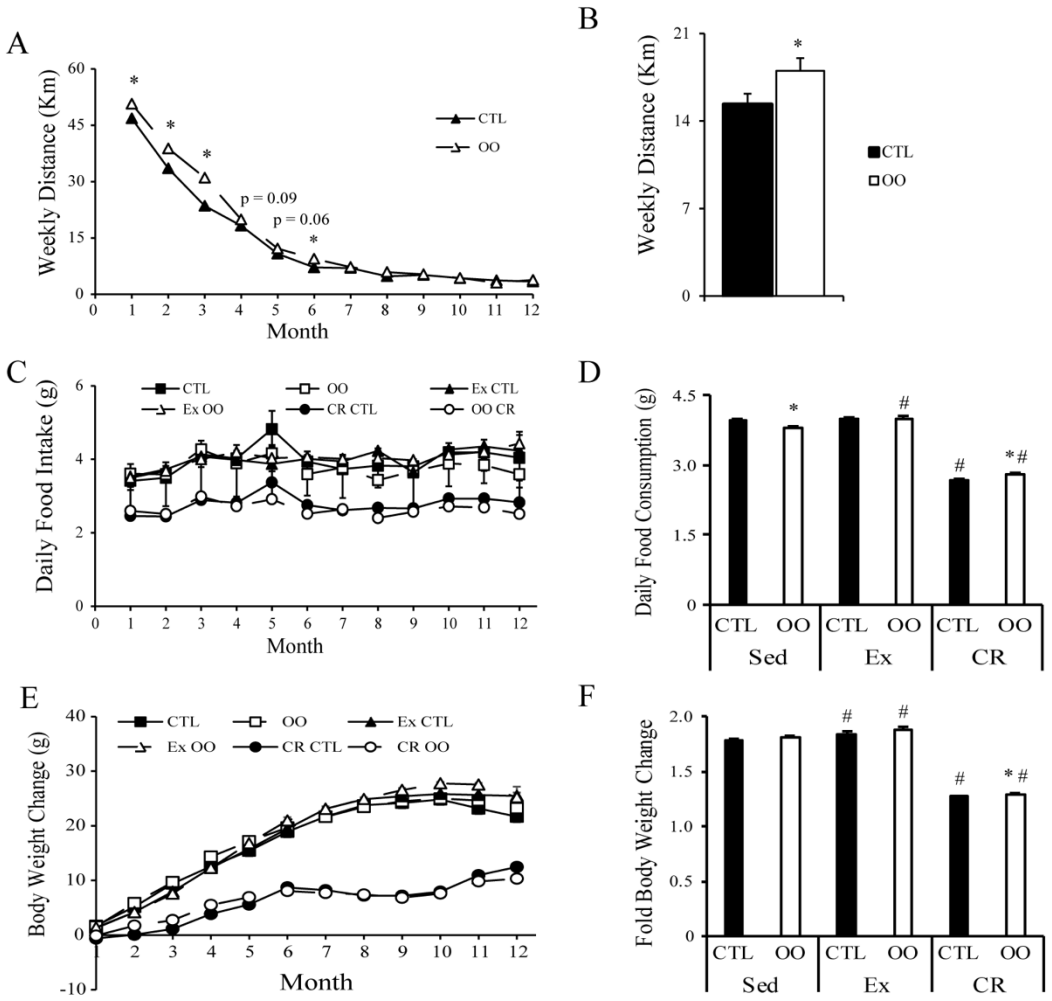
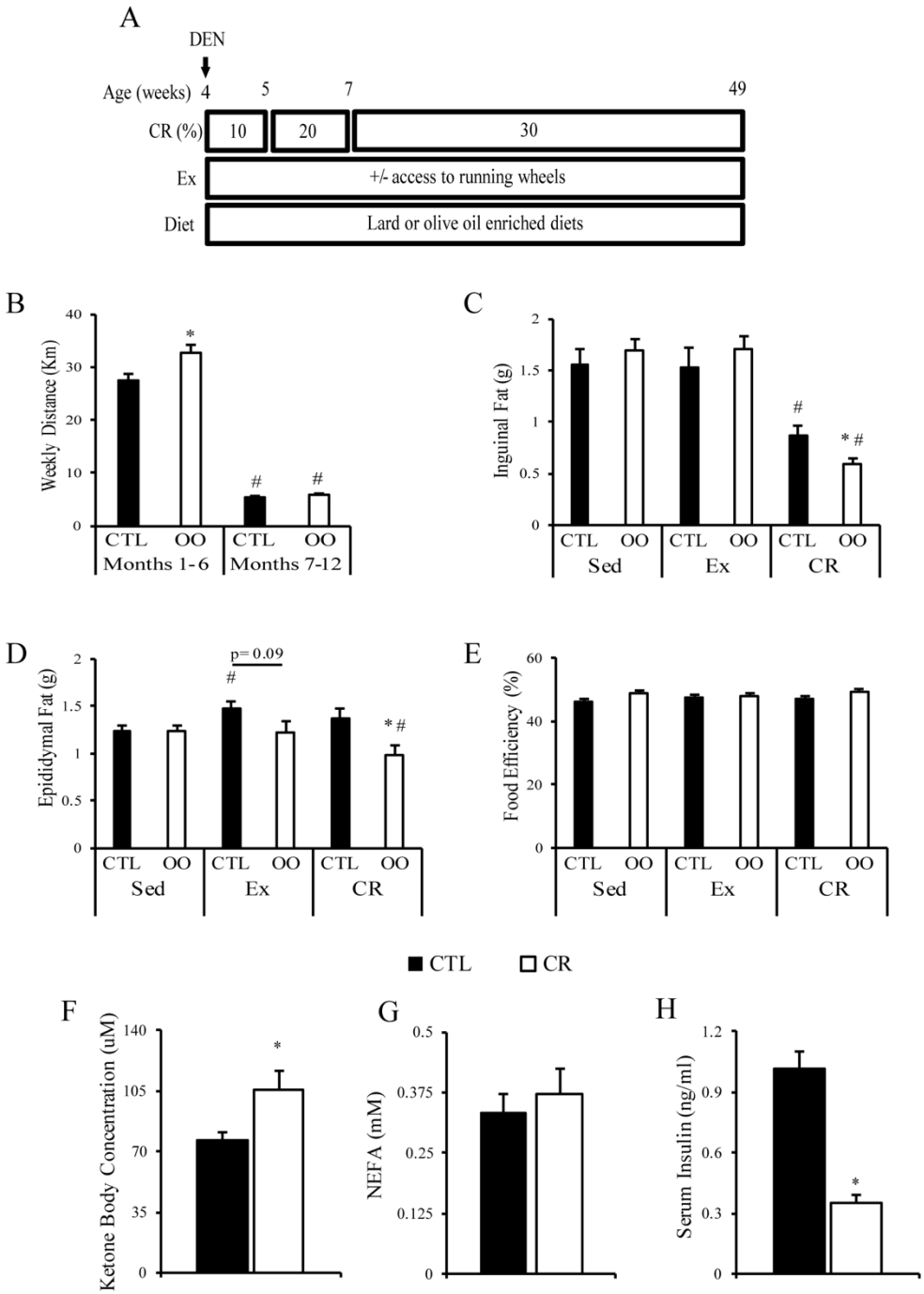


Figure 1. Phenotypic responses to treatment regiments. (A) Average weekly running distance for each month of the study and (B) collective average running distance of OO and CTL fed mice. (C) Average daily food intake by month and (D) average daily food consumption over the course of the study. (E) Monthly body weight change and (F) average total body weight change. Data are presented as ± SEM. *P<0.05 compared to CTL, #P<0.05 compared to Sed.


A
DEN
Age (weeks) 4
5
7
49
CR (%)
10
20
30
Ex
+/- access to running wheels
Diet
Lard or olive oil enriched diets
B
Weekly Distance (Km)
CTL OO CTL OO
Months 1-6 Months 7-12
C
Inguinal Fat (g)
CTL OO CTL OO CTL OO
Sed Ex CR
D
Epididymal Fat (g)
p= 0.09
CTL OO CTL OO CTL OO
Sed Ex CR
E
Food Efficiency (%)
CTL OO CTL OO CTL OO
Sed Ex CR
CTL
CR
F
Ketone Body Concentration (uM)
G
NEFA (mM)
H
Serum Insulin (ng/ml)

Supplemental Figure 1. Phenotypic and metabolic parameters are influenced by Ex and CR. (A) Schema of the study protocol. Four-week-old C57BL/6 mice were given an intraperitoneal injection of DEN at 25 mg/kg body weight. Mice were subsequently randomly stratified into two dietary groups (CTL or OO) and then again into one of three groups: Sed, Ex and CR. To preserve normal development, 30% macronutrient restriction was achieved stepwise over the course of 3 weeks; all mice were individually housed. (B) Weekly distance parceled into 6 month increments. (C & D) Epididymal and inguinal fat pad weights. (E) Food efficiency analysis. (F) Serum ketone body concentrations analyzed from CTL and CR mice. (G) Non-esterified fatty acids (NEFA) analyzed in serum from CTL and CR mice. (H) Serum insulin analysis of CTL and CR mice. Data are presented as ± SEM. *$P<0.05$ compared to CTL, $^{\#}P<0.05$ compared to Sed.

Caloric Restriction Prevents Hepatic Tumorigenesis

Upon termination of the study, 49 weeks after DEN administration, liver surface nodules were quantified. Tumors were present in approximately half of mice in the Sed and Ex groups irrespective of diet (Fig. 2A). However, no surface nodules were observed in any mice in the CR groups. Clinical diagnosis of tumors was confirmed by a blinded pathologist from H&E stained slides and demonstrated a diversity of hepatic tumors including hepatocellular carcinomas, hepatoblastomas, adenomas, and cholangiocarcinomas. Pearson's correlation analysis demonstrated a significant positive correlation between body weight change or food intake and tumor number (Fig. 2B-C), while activity level was not correlated to surface nodules (Fig. 2D).

Because no differences in tumor formation were observed between dietary or exercise interventions, subsequent analyses were largely focused on the differences between Sed and CR groups fed the CTL diet. Consistent with resistance to tumor formation, non-transformed livers of CR mice showed an ~85% reduction in staining of the proliferative marker Ki-67 (Fig. 2E). Collectively, this data demonstrates CR robustly protects mice from hepatic tumorigenesis initiated by DEN and promoted through *ad libitum* caloric intake.

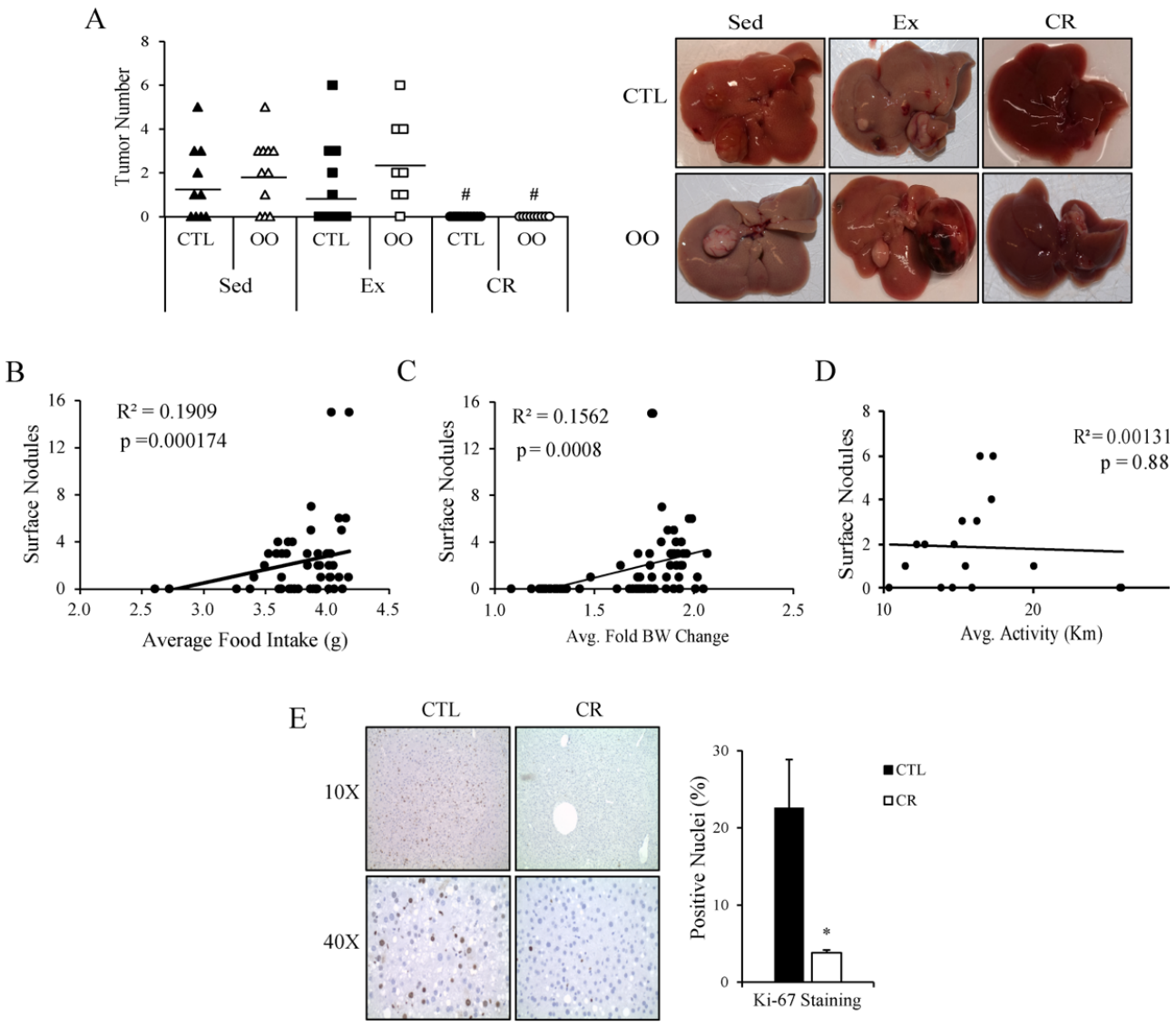


Figure 2. CR prevents hepatic tumor formation. (A) Scatter plot of quantified visible surface nodules per mouse and representative images of livers from mice from each group; horizontal bars represent group mean. (B-D) Pearson's correlation analysis of average food intake, average fold body weight change, and average weekly running wheel activity with surface nodule count. (E) Representative 10X and 40X images from 5 randomly selected views per

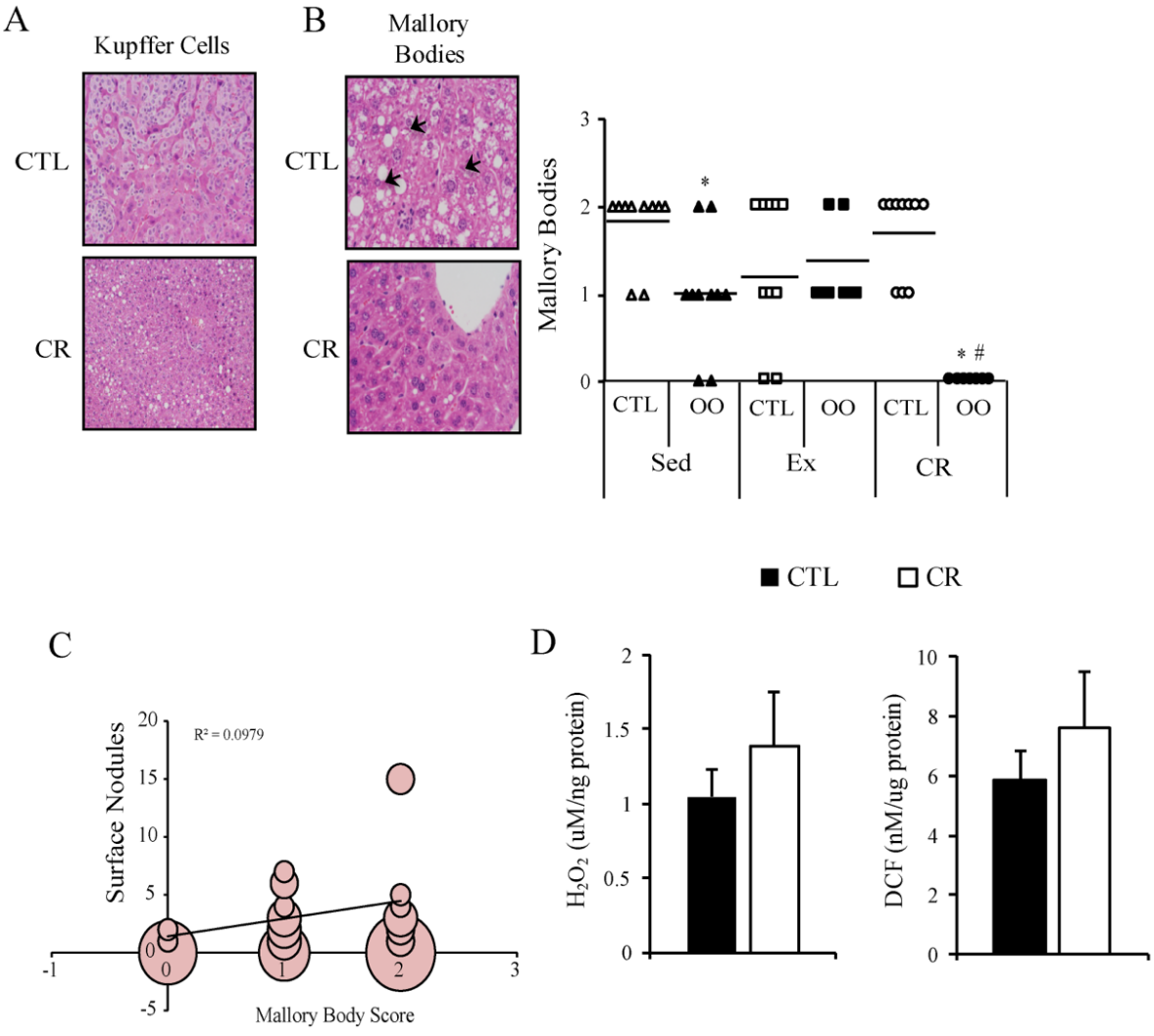
animal from non-transformed tissue of CTL and CR mice and quantification of positively stained nuclei. Data are presented as ± SEM. *P<0.05 compared to CTL, #P<0.05 compared to Sed.

Caloric Restriction Prevents Pathologies Associated with NAFLD-Driven Liver Cancer

A recent model describing the progression of NAFLD to liver cancer suggests that steatosis, inflammation and cellular damage may act simultaneously rather than in succession in the development of liver cancer (255). In this context, CR has been shown to improve hepatic lipid metabolism and reduce hepatic inflammation (256). Indeed, compared to their dietary CTL, liver weights were significantly lower in CR mice, but higher in the Ex mice (Fig. 3A). The OO diet increased liver weights within the Ex group, but lowered them in response to CR (Fig. 3A). Livers from CR mice showed reduced lipid droplet accumulation as visualized with H&E staining, which was confirmed with Oil Red O staining (Fig. 3B & C). These data were further confirmed via steatosis scoring of H&E slides performed by a blinded pathologist (Fig. 3D) demonstrating that CR prevented steatotic burden. In addition, serum ketone body concentrations were significantly increased in CR mice (Fig. S1F) in the absence of changed serum non-esterified fatty acids (Fig. S1G) and reduced insulin (Fig. S1H), suggesting reduced steatosis in these mice is partially explained by increased oxidation of fatty acids. Pearson's correlation analysis showed a strong positive relationship

between steatosis with tumor burden (Fig. 3E) supporting the established link between NAFLD and hepatic tumorigenesis (257–259).

In addition to abrogating steatotic burden, CR also reduced markers of immune cell infiltration. Staining of neutrophils (Fig. 4A) and CD3 T-cells (Fig. 4B) and the presence of Kupffer cells (Fig. S2A) was reduced in livers of CR mice. Consistent with reduced immune cells, CR significantly attenuated lobular inflammation (Fig. 4C), hepatocyte ballooning (Fig. 4E) and the presence of Mallory bodies (Fig. S2B). The pathological scores associated with lobular inflammation and ballooning, but not Mallory bodies, correlated with tumor burden (Fig. 4D and F and Fig. S2C). Despite the frequent association of inflammation with ROS, there were no differences in hydrogen peroxide or total reactive oxygen species in liver tissues of CR mice (Fig. S2D). Collectively, this data show that CR reduces hepatic steatosis, inflammation, cellular damage, and immune cell infiltration, markers associated with NAFLD progression that correlate to hepatic tumorigenesis.


A
Kupffer Cells
CTL
CR
B
Mallory Bodies
CTL
CR
Mallory Bodies
3
2
1
0
*
* #
CTL
OO
CTL
OO
CTL
OO
Sed
Ex
CR
CTL
CR
C
Surface Nodules
R² = 0.0979
20
15
10
5
0
-5
-1
3
0
0
1
2
Mallory Body Score
D
H2O2 (uM/ng protein)
2
1.5
1
0.5
0
DCF (nM/ug protein)
10
8
6
4
2
0

Supplemental Figure 2. CR modulates additional markers of hepatic inflammation and immune cell infiltration.

(A) H&E images revealed differences in Kupffer cell presence in CTL and CR mice. (B) Mallory bodies from H&E staining of representative 40X images from 5 randomly selected views per animal and blinded pathologist scoring of lobular inflammation. (C) Pearson's correlation of Mallory bodies. (D) Quantification of Hydrogen Peroxide and other non-hydrogen peroxide free radicals (DCF) found in non-transformed tissue from CTL and CR mice.

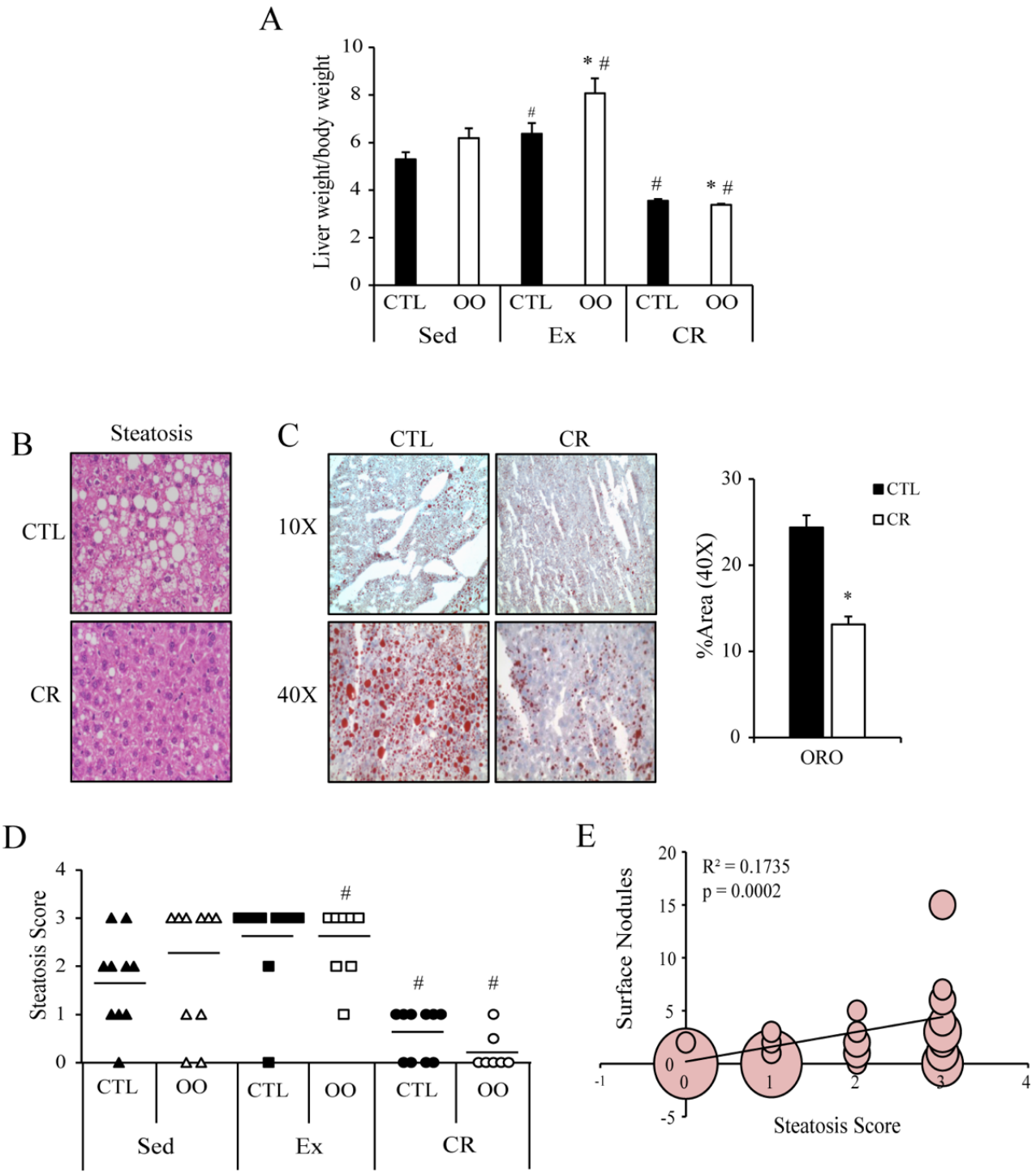

A
Liver weight/body weight
CTL
OO
Sed
Ex
CR
B
Steatosis
C
10X
40X
%Area (40X)
ORO
D
Steatosis Score
E
R² = 0.1735
p = 0.0002
Surface Nodules
Steatosis Score

Figure 3. CR prevents hepatic steatosis.

Liver weight to body weight ratio (A) of mice (n=57) and representative H&E images (B) of livers from CTL and CR mice. (C) Oil Red O staining and quantification from representative 10X and 40X images from 5 randomly selected views per animal from non-transformed tissue of CTL and CR mice and quantification of average lipid stained area per field. (D) Steatosis scoring assigned to each representative case as performed by a pathologist (J.C.M.) blinded to treatments; horizontal bars represent group means (E) Pearson's correlation of steatosis score with surface nodules. For E; larger data points represent multiple mice falling with the same nodule/score intersection. Data are presented as ± SEM. *$P<0.05$ compared to CTL, $^{\#}P<0.05$ compared to Sed.

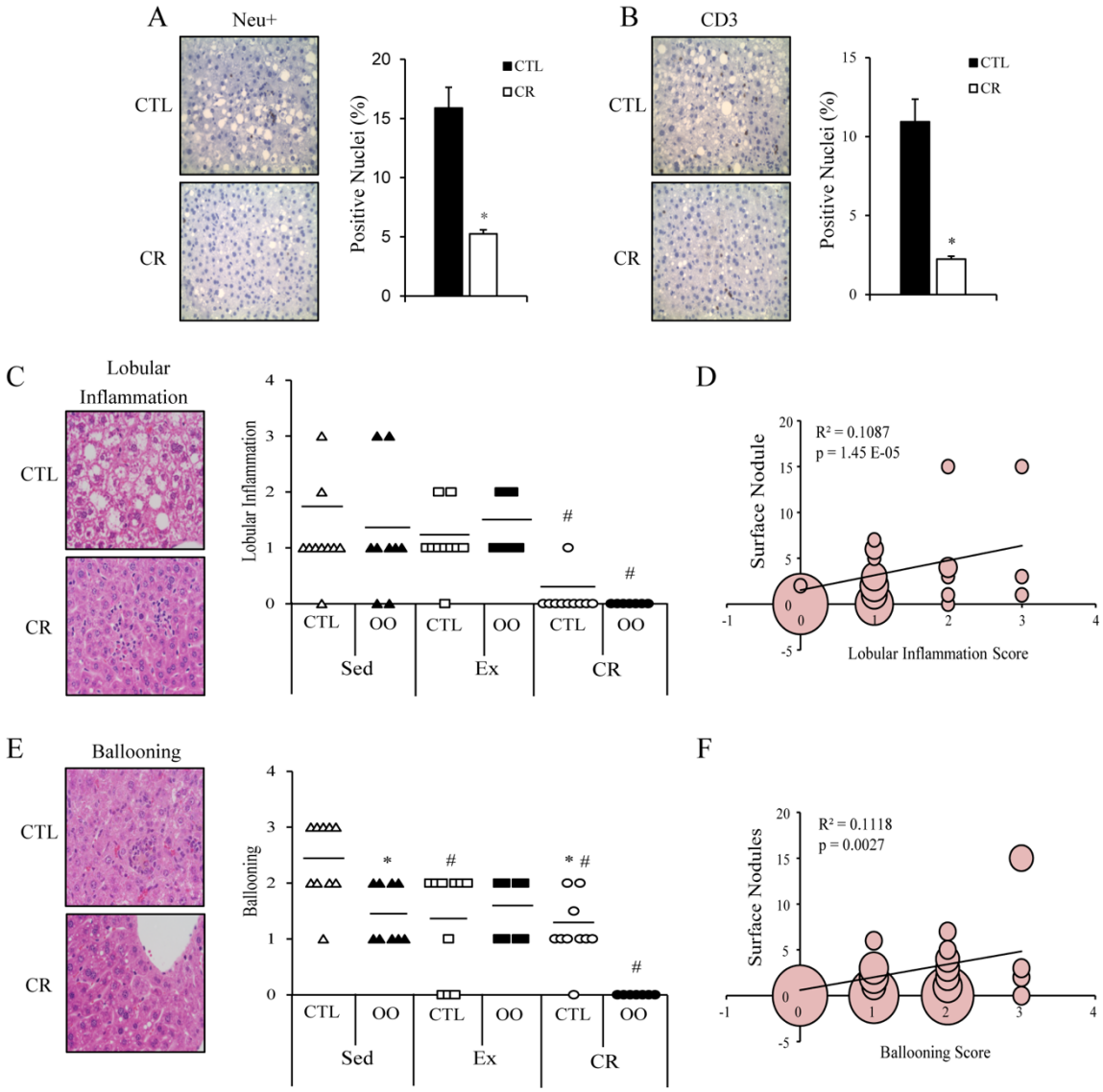

A
Neu+
CTL
CR
Positive Nuclei (%)
CTL
CR
B
CD3
C
Lobular Inflammation
Lobular Inflammation
Sed
Ex
CR
D
$R^2 = 0.1087$
$p = 1.45$ E-05
Surface Nodule
Lobular Inflammation Score
E
Ballooning
Ballooning
F
$R^2 = 0.1118$
$p = 0.0027$
Surface Nodules
Ballooning Score

Figure 4. Caloric restriction abrogates pathologies associated with NAFLD progression.

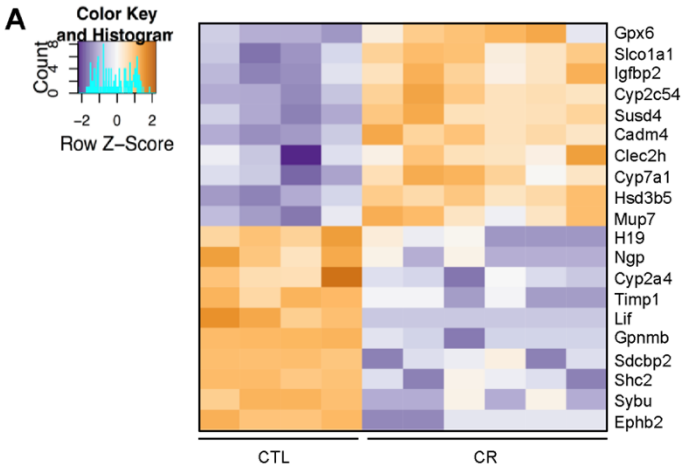
(A) Immunohistochemical staining of neutrophils from representative 40X images from 5 randomly selected views per animal from non-transformed tissue of CTL and CR mice and quantification of positively stained nuclei. (B) Immunohistochemical staining of CD3+ T-cells from representative 40X images from 5 randomly selected views per animal from non-transformed tissue of CTL and CR mice and quantification of positively stained nuclei. (C) Lobular inflammation from H&E staining of representative 40X images from 5 randomly selected views per animal and blinded pathologist scoring of lobular inflammation. (D) Pearson's correlation of lobular inflammation to surface nodules. (E) Hepatocellular ballooning from H&E staining of representative 40X images from 5 randomly selected views per animal and blinded pathologist scoring of lobular inflammation. (F) Pearson's correlation of ballooning to surface nodules. For D-E; larger data points represent multiple mice falling with the same nodule/score intersection. Data are presented as ± SEM. *$P<0.05$ compared to CTL, $^{\#}P<0.05$ compared to Sed CTL.

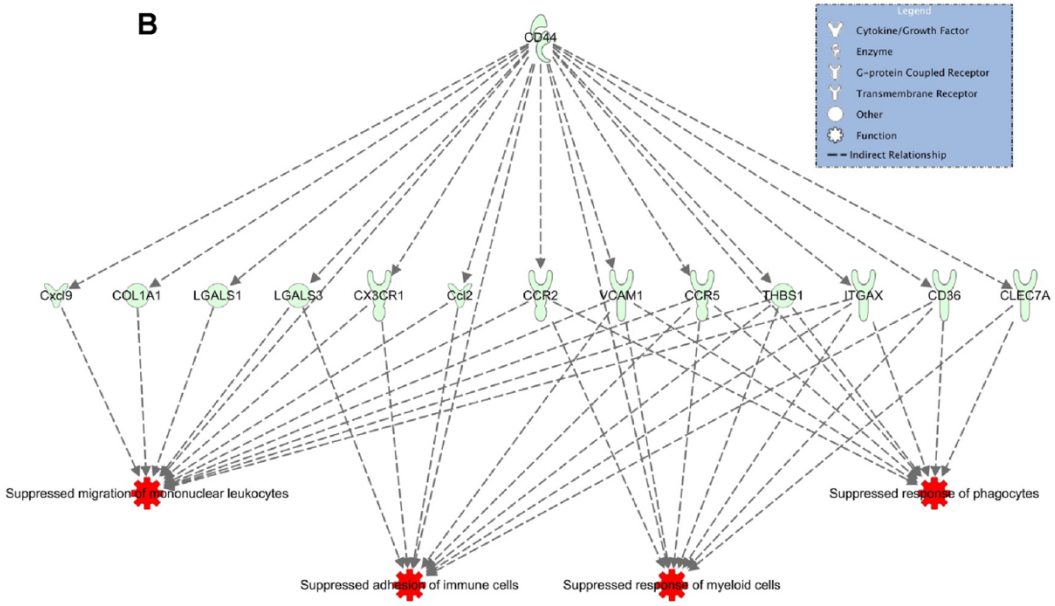
Non-Transformed Liver Tissue from CR and CTL Mice Produce Significantly Distinct Gene Profiles

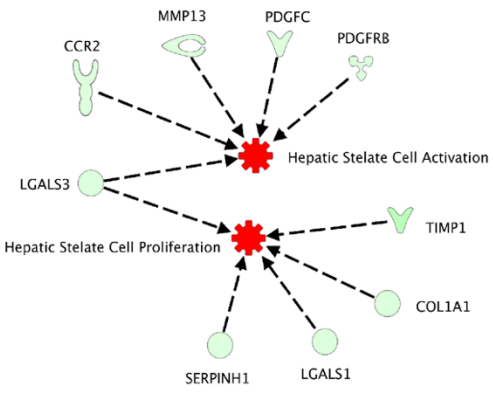
Because CR mice developed no visible tumors, transcriptome profiling of non-transformed tissue was performed to elucidate potential mechanisms underlying the carcinogenic process in our model. Initial analysis demonstrated a total of 487 differentially expressed genes (Fig. S3A) with the top 20 up/downregulated genes listed in (Fig. 5A). To better understand the biological relevance of the observed differences in the gene signature profiles of CR and CTL mice, multiple parameters were analyzed using Ingenuity Pathway Analysis (IPA). IPA's initial assessment identified a total of 223 canonical pathways as being significantly changed between CR and CTL groups. Interestingly, of the10 most significantly impacted pathways, 9 were related to hepatic inflammation, immune cell activation, and hepatic fibrosis (Table 1); these data corroborated the histological analyses (Fig. 4). Further investigation of the identified pathways elucidated cell adhesion/movement (Fig. 5B), toll-like receptor signaling (Fig. S4A), lipid/carbohydrate metabolism (Fig. S4B), and cell growth and proliferation (Fig. S4B) as regulatory networks relevant to transcriptomic alterations. From this information, IPA predicted suppression of several features relevant in the NAFLD progression to liver cancer including hepatic stellate cell activation and proliferation (Fig. 5C) and HCC (Fig. 5D).

Because gene signatures, canonical pathways, and downstream networks/signaling pathways corroborated previous data linking NAFLD and subsequent pathologies such as inflammation and cellular damage leading to

non-alcoholic steatohepatitis (NASH), IPA was used to elucidate downstream effects of pathway and network influences. Initial analysis identified 188 biologic functions, diseases, and toxological outcomes related to our identified differentially expressed gene profiles. The most significant diseases and biological functions were categorized by steatosis, liver damage, liver necrosis, hepatocellular adhesion and fibrosis, hepatic hypertrophy and hyperplasia, and liver cancer; CR robustly altered gene profiles attenuating these pathologies in comparison to CTL mice (Table 2). Importantly, these gene signatures matched the biochemical and histological profiles previously identified (Fig. 4 and 5). Collectively, these results suggest CR leads to unique transcriptomic alterations, which are relevant to numerous canonical pathways and gene networks associated with NAFLD progression to liver cancer.


A
Color Key and Histogram
Count
Row Z-Score
Gpx6
Slco1a1
Igfbp2
Cyp2c54
Susd4
Cadm4
Clec2h
Cyp7a1
Hsd3b5
Mup7
H19
Ngp
Cyp2a4
Timp1
Lif
Gpnmb
Sdcbp2
Shc2
Sybu
Ephb2
CTL
CR


B
CD44
Legend
Cytokine/Growth Factor
Enzyme
G-protein Coupled Receptor
Transmembrane Receptor
Other
Function
Indirect Relationship
Cxcl9
COL1A1
LGALS1
LGALS3
CX3CR1
Ccl2
CCR2
VCAM1
CCR5
THBS1
ITGAX
CD36
CLEC7A
Suppressed migration of mononuclear leukocytes
Suppressed adhesion of immune cells
Suppressed response of myeloid cells
Suppressed response of phagocytes


C
MMP13
PDGFC
PDGFRB
CCR2
Hepatic Stelate Cell Activation
LGALS3
TIMP1
Hepatic Stelate Cell Proliferation
COL1A1
SERPINH1
LGALS1

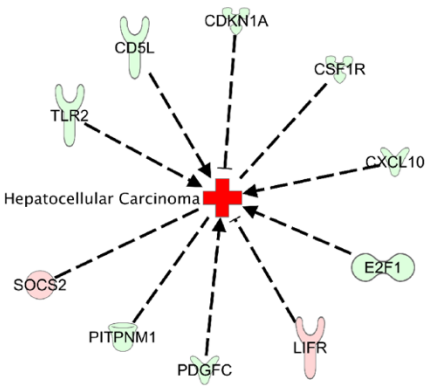
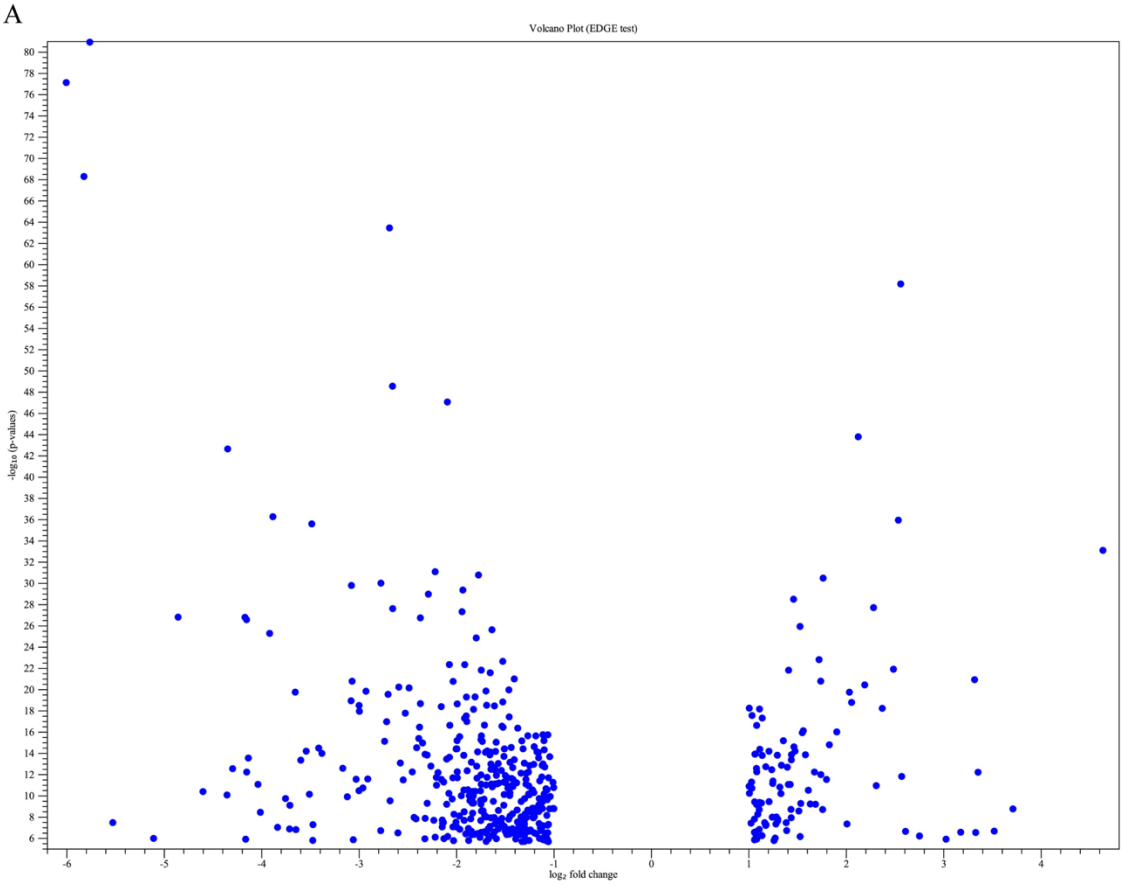

D
CDKN1A
CD5L
CSF1R
TLR2
CXCL10
Hepatocellular Carcinoma
E2F1
SOCS2
PITPNM1
LIFR
PDGFC

Figure 5. Caloric restriction produce significantly distinct gene profiles from control mice.

(A) Heat map of the top 10 most upregulated and 10 most downregulated genes. (B) Cell adhesion/migration pathway was identified as a major signaling pathway by IPA. (C) IPA identification of hepatic stellate cell activation and hepatic stellate cell proliferation as top biological functions. (D) IPA identification of hepatocellular carcinoma as the most significant disease associated with gene signatures. For (B-D) Green targets indicate lower expression in CR compared to CTL and red indicate increased expression; table in (B) corresponds to (B-D).

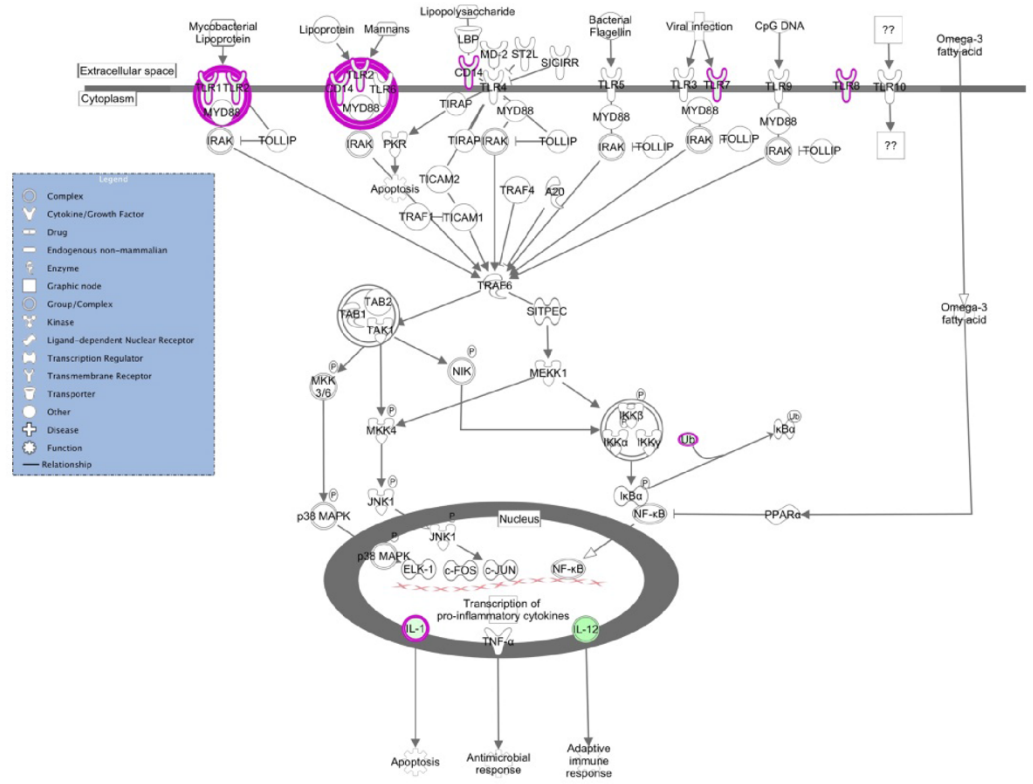
A



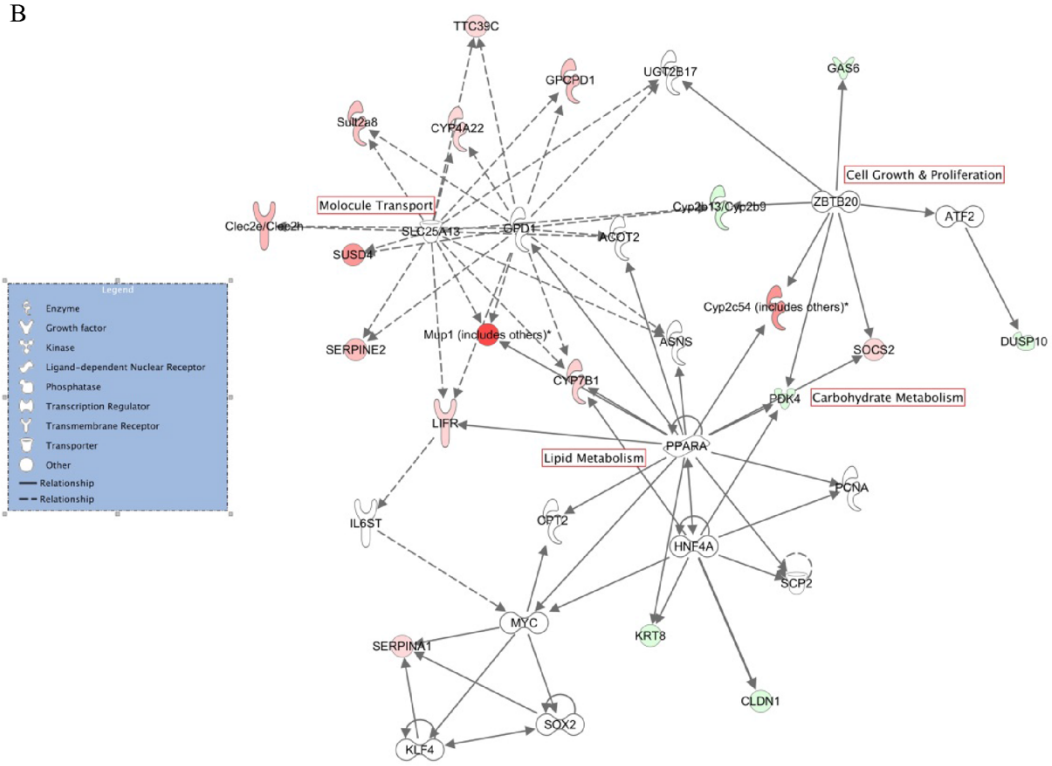
Supplemental Figure 3. CR causes divergence of genes in comparison to CTL mice.

(A) Volcano plot demonstrating the pattern of divergence and the fold induction of the 487 identified genes in the data set.

A


Mycobacterial Lipoprotein
Lipoprotein
Mannans
Lipopolysaccharide
LBP
MD-2 ST2L
CD14
SIGIRR
Bacterial Flagellin
Viral infection
CpG DNA
??
Omega-3 fatty acid
Extracellular space
Cytoplasm
TLR1 TLR6
TLR2
CD14
TLR4
TLR5
TLR3
TLR7
TLR9
TLR8
TLR10
MYD88
TIRAP
IRAK
TOLLIP
PKR
Apoptosis
TICAM2
TRAF4
A20
TRAF1
TICAM1
TRAF6
TAB2
TAB1
TAK1
SITPEC
MKK 3/6
NIK
MEKK1
MKK4
IKKβ
IKKα
IKKγ
Ub
IκBα
JNK1
p38 MAPK
NF-κB
PPARα
Nucleus
ELK-1
c-FOS
c-JUN
Transcription of pro-inflammatory cytokines
IL-1
IL-12
TNFα
Antimicrobial response
Adaptive immune response
Legend
Complex
Cytokine/Growth Factor
Drug
Endogenous non-mammalian
Enzyme
Graphic node
Group/Complex
Kinase
Ligand-dependent Nuclear Receptor
Transcription Regulator
Transmembrane Receptor
Transporter
Other
Disease
Function
Relationship

B


TTC39C
GPCPD1
UGT2B17
GAS6
Sult2a8
CYP4A22
Cell Growth & Proliferation
Molecule Transport
Clec2e/Clec2h
SLC25A13
CPD1
ACOT2
Cyp2b13/Cyp2b9
ZBTB20
ATF2
SUSD4
Cyp2c54 (includes others)*
SERPINE2
Mup1 (includes others)*
ASNS
SOCS2
DUSP10
CYP7B1
PDK4
Carbohydrate Metabolism
LIFR
PPARA
Lipid Metabolism
PCNA
IL6ST
CPT2
HNF4A
SCP2
MYC
KRT8
SERPINA1
CLDN1
SOX2
KLF4
Legend
Enzyme
Growth factor
Kinase
Ligand-dependent Nuclear Receptor
Phosphatase
Transcription Regulator
Transmembrane Receptor
Transporter
Other
Relationship
Relationship

Supplemental Figure 4. CR affects gene signature pathways and networks associated with hepatic inflammation and metabolism.

(A) IPA analysis identification of the Toll-like receptor pathway as a significantly downregulated pathway in CR mice compared to CTL; purple highlights indicate multiple targets confined within highlighted category and green color of intermediates indicates downregulated targets in CR compared to CTL. (B) IPA analysis identification of a network involved in lipid metabolism, carbohydrate metabolism, and cell growth and proliferation; green color indicates downregulated genes compared to CTL and red indicates upregulated genes. Darker color indicates greater up/downregulation.

Table 1. The 10 most significant canonical pathways in NT hepatic tissue comparing CR to CTL group.

Ingenuity Canonical Pathways	-log (p-value)	Ratio (numerical)	Genes
Hepatic Fibrosis & Hepatic Stellate Cell Activation	7.46	17/117 (0.145)	*CCR5, VCAM1, ICAM1, COL4A1, COL12A1, KLF6, PDGFC, COL1A2, COL1A1, COL6A3, TIMP1,* ***CYP2E1****, CD14, SERPINE1,* ***COL27A1****, COL3A1, PDGFRB*
Atherosclerosis Signaling	7.43	14/78 (0.179)	*COL1A2, ITGB2, COL1A1, VCAM1, APOA4, ICAM1, IL1RN, CXCR4, CD36, LPL,* ***SERPINA1****, PLA2G7, PDGFC, COL3A1*
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	7.09	11/48 (0.229)	*TLR2, HLA-A, IL1RN, TLR1, HLA-DMB, TLR8, HLA-DQA1, TLR7, Tlr13, HLA-DQB1, Tlr12*
Communication between Innate and Adaptive Immune Cells	5.54	9/43 (0.209)	*CXCL10, TLR2, HLA-A, IL1RN, TLR1, TLR8, TLR7, Tlr13, Tlr12*
B Cell Development	5.11	5/11 (0.455)	*PTPRC, HLA-A, HLA-DMB, HLA-DQA1, HLA-DQB1*
Dendritic Cell Maturation	4.51	12/101 (0.119)	*TLR2, COL1A2, COL1A1, ICAM1, HLA-A, IL1RN, TYROBP, TREM2, HLA-DMB, HLA-DQA1, HLA-DQB1, COL3A1*
TREM1 Signaling	4.09	8/50 (0.16)	*TLR2, ICAM1, TYROBP, TLR1, TLR8, TLR7, Tlr13, Tlr12*
LPS/IL-1 Mediated Inhibition of RXR Function	4.07	15/166 (0.0904)	*ALDH1B1,* ***SLC10A1****, GSTA5, ALAS1,* ***GSTT2/GSTT2B****, Cyp2a12/Cyp2a22, CYP3A5, Gstm3, IL1RN,* ***NR1I3****,* ***CYP7A1****, FABP4, CD14, CYP2A6 (includes others),* ***CYP4A11***
Antigen Presentation Pathway	4.05	5/17 (0.294)	*HLA-A, HLA-DMB, HLA-DQA1, HLA-DQB1, CD74*

Genes in bold are up-regulated

Table 2. The 11 most significant categories of disease/toxological function in NT hepatic tissue comparing CR to CTL group.

Categories	Disease/Function	p-value	Genes	# of Molecules
Liver Damage	Injury of liver	9.35E-08	*Ccl2, CCR5, CD14, CDKN1A, CXCL10,* ***CYP2E1, CYP7A1****, HLA-A, IL1RN, ITGB2, KRT8, LIF, NT5E,* ***SERPINA1****, SERPINE1,* ***SLC10A1****, TLR2*	17
Liver Damage	Damage of liver	4.63E-07	*Ccl2, CCR5, CD14, CD44, CDKN1A, CXCL10,* ***CYP2E1, CYP7A1****, HLA-A, IL1RN, ITGB2, KRT8, LIF, NR1I3, NT5E,* ***SERPINA1****, SERPINE1,* ***SLC10A1****, TLR2*	19
Liver Necrosis & Cell Death	Necrosis of liver	5.21E-05	*CD14, CDKN1A, CXCL10,* ***CYP2E1, CYP7A1****, E2F1, GADD45B, IL1RN, ITGB2, KRT8, LGALS3, LIF,* ***NR1I3****, PTPRC, SERPINE1, TIMP1, TLR7, TYROBP*	18
Liver Adhesion	Adhesion of hepatocytes	2.27E-04	*ICAM1, KRT8, VCAM1*	3
Liver Fibrosis	Fibrosis of liver	2.57E-04	*COL1A1, COL1A2, HSPB1, IL2RG, LGALS3, PDGFC, PDGFRB, SERPINE1, TIMP1, Ccl2, CXCL10, LGALS3, PDGFC, PDGFRB, TIMP1*	15
Liver Cholestasis	Progressive familial intrahepatic cholestasis type 2	4.45E-04	***CYP7A1, SLC10A1****, SLC10A2*	3
Liver Inflammation	Inflammation of liver		*CD14, CD44, CYP2E1, HLA-A, IL1RN, ITGB2, LGALS3, PTPRC, SERPINE1,* ***SLC10A1****, SPI1, TLR7*	12
Liver Necrosis & Cell Death	Cell death of liver cells	1.22E-03	*CDKN1A, CXCL10,* ***CYP2E1, CYP7A1****, E2F1, GADD45B, IL1RN, ITGB2, KRT8, NR1I3, PTPRC, TIMP1, TLR7*	13
Liver Cholestasis	Hepatic cholestasis	1.62E-03	*CD68, CDKN1A, CYP7A1, RDH16, SLC10A1, SLC10A2,* ***Slco1a1***	7
Liver Hypertrophy	Hypertrophy of liver	1.94E-03	***CYP1A2, NR1I3***	2
Liver Steatosis	Hepatic steatosis	4.79E-03	*Ccl2, CCR5, CD14,* ***CYP2E1****, INSIG2,* ***NR1I3****, PDGFC, TLR2*	8

Genes in bold are up-regulated.

Discussion

Epidemiological and experimental data has long supported CR as a preventative intervention against numerous forms of cancer. CR studies have both focused on increasing longevity and delaying tumorigenesis in aged models (260), in improving disease free longevity (261), and limiting tumor progression (262). The present study demonstrates CR serves as a potent deterrent of hepatic carcinogenic promotion and progression. The results from this study both confirm previous reports demonstrating the longevity and anti-carcinogenic properties of CR and expand upon them, characterizing pathologic and transcriptome alterations induced by CR, thus providing mechanistic insights as to how CR elicits its oncogenic protection.

Etiologies of liver cancer vary greatly between developed and developing countries. In developed regions of the world, obesity and subsequent NAFLD progression, has been well described to contribute to increase the risk of hepatocellular carcinoma and other liver cancers. In this study, CR significantly reduced steatotic burden, which strongly correlated with tumor risk. These data support previous studies showing that CR can reverse steatosis in obese rats (263). However, simple steatosis is not in and of itself pathologic. Instead, it is the subsequent damage resultant of continued neutral lipid accumulation in combination with derangements, which often accompany severe obesity, that play a pathologic role in hepatic cancer risk. NASH, which is characterized by increased inflammation and cell damage greatly increases the risk in development of liver cancer (211, 263). In fact, population estimates from clinical

studies suggest NASH patients are twice as likely to develop cirrhosis when compared to hepatitis C patients (264). Demonstrating the importance of limiting progression of NAFLD, CR has been shown to curb the development of NASH (264). Corroborating this feature, CR mice had reduced lobular inflammation and hepatocyte ballooning. Resident hepatic macrophages known as Kupffer cells play an important role in mediating the response to liver injury (265). Additionally, the innate immune response plays an important role in mediating acute inflammation and hepatocyte damage (266). However, in NAFLD and NASH, chronic inflammation results in immune cell activation and infiltration, which promotes liver injury (267). Most notably, this aberrant response sensitizes toll-like receptor signaling (268), which among other effects, increases production of inflammatory cytokines such as TNF-α, IL-12, and NF-κB (269). Histological and IHC analysis of CR mice exhibited reduced Kupffer cells, CD3+ T-cells, and neutrophils. Additionally, RNA-sequencing data showed that CR mice had reduced expression of genes encoding transcription factors, cytokines, and receptors involved in inflammation, liver damage, necrosis, and immune cell activation and infiltration. Thus, CR robustly prevents alterations of gene signatures, effectors, signaling networks, and subsequent disease development involved in the progression of NAFLD to liver cancer.

A major consequence of progressive NASH, and risk factor for cirrhosis, is the development of fibrosis (264). Fibrosis is most notably accomplished through Kupffer cell and inflammatory cytokine activation of hepatic stellate cells. Located in the space of Disse, hepatic stellate cells serve as the primary source of

extracellular matrix proteins that contribute to fibrosis (270). TLR4 signaling initiates hepatic stellate cells to produce chemokines (CCL family), leading to recruitment of Kupffer cells, and increased TGF-β signaling (271). At the same time, TLR9 signaling initiates hepatic stellate cell collagen production, further progressing fibrosis (272). Hepatic stellate cell activation and proliferation were two major biologic functions found to be downregulated in CR mice through IPA of RNA-seq data. Additionally, *TLR4 and TLR9* were both downregulated as were numerous CCL chemokines in CR mice, but only *TLR9* and *CCL1, 2, 20,* and *22* met our cutoff parameters for statistical significance. This data supports previous reports investigating the effects of long-term CR on NF-κB signaling (273) and other inflammatory cytokines (274). The development of fibrosis and cirrhosis are established risk factors for the development of liver cancers including HCC (275). In accordance with this, IPA identified several genes related to the development of HCC with most downregulated genes in this network being positively associated with HCC (276), while those that were upregulated in CR mice are identified liver tumor suppressors (277). Within the hepatic tumor microenvironment, cell adhesion and migration in part driven by CD44, plays a critical role in the invasiveness of tumors (278). IPA determined CD44 mediated migration of pro-inflammatory molecules was universally repressed, identifying CR as a mitigator of cell damage leading to tumorigenesis. Collectively, this data highlights CR as a robust deterrent of signaling cascades and downstream biologic consequences associated with hepatic tumorigenic etiologies relevant to developed countries.

As a staple of the Mediterranean Diet, extra virgin OO elicits numerous health benefits. Previous studies have shown that the polyphenolic compounds in OO have anti-inflammatory and anti-oncogenic effects (279). However, these compounds are greatly enriched in extra virgin olive oil and variable amongst oils. Analyses of these polyphenolic compounds in our diets revealed undetectable levels suggesting poor phenolic content of our OO diet could potentially explain a lack of dietary effect on tumorigenesis.

Independent evidence has shown that exercise and the MD reduce liver cancer risk, yet no studies have compared the synergistic effects of exercise and specific dietary constituents of the MD. Since mice have been documented to readily utilize running wheels (280), we used voluntary wheel running to test the effects of exercise on liver oncogenesis. Although mice initially utilized running wheels, after ~5 months activity levels did not exceed reported ambulatory movement (281). Lack of activity is further apparent when assessing body weight, which increased as running wheel utilization diminished. Given that phenotypic changes of Ex mice mirrored that of CTL mice, it is not surprising there were no differences in tumorigenesis. Future studies of this nature should use controlled exercise interventions to account for individual variability in activity level and to ensure adherence of treatment for the duration of the study.

Overall, these findings demonstrate CR as a robust deterrent of liver cancer and provide insight to possible mechanisms related to the pathology of liver cancer. Liver cancer is among the fastest growing cancers, especially in men, in developed countries and it remains among the world's deadliest cancers with

mortality rates as high as 61% within 1 year of diagnosis. Given the limited treatment options, results here provide promise in describing CR or related dietary regiments as a therapeutic tool in cancer prevention and advance our understanding into the molecular mechanisms underlying the anti-oncogenic effects of CR.

Chapter 4

Perspectives and Future Directions

Jonathan M Ploeger wrote this chapter in its entirety

Obesity continues to be one of the most prominent chronic health issues facing developed countries. Currently, over 70% of adults are considered overweight or obese while nearly 21% of children ages 12-19 are obese (282). More troubling, the proportion of children with obesity continues to increase with age suggesting this troubling trend will continue to plague Western societies for years to come with continued implementation of current therapeutic strategies. Economically, obesity continues to be one of the most expensive chronic conditions, costing Americans between $147 and $210 billion dollars annually in direct treatment costs associated with obesity and related diseases (283). However, the impact of obesity reaches beyond the bedside. Absenteeism resultant from obesity reached an estimated 450 million hours of lost work which translated into nearly $153 billion dollars in lost wages (284).

Obesity has long been linked to numerous other pathologies including NAFLD. However, only in the past several years has obesity and NAFLD been identified as major risk factors for many different types of cancer. As a testament to this relationship, reduced cancer-related deaths are the primary factor contributing to the lower mortality in obese subjects that have undergone bariatric surgery (285). Moreover, the risk for HCC-related mortality is increased ~4.5 fold in obese males, more than any other type of cancer (29).

Liver cancer has become a major health problem in developed countries, including the United States. Currently it is the fifth most common and one of the deadliest forms of cancer worldwide with more than 86% of cases resulting in death (286, 287). The only viable option to treat liver cancer is partial liver

removal. In the setting of obesity, partial liver removal/liver transplantation is often not indicated due to poor prognosis following surgery. In addition to dramatically rising incidence of HCC, the mortality rate for NAFLD derived HCC is higher compared to other etiologic factors such as hepatitis viral infections, reaching as high as 61% mortality within 1 year of diagnosis (288). Furthermore, current treatment strategies are limited and largely palliative, thus demonstrating an urgent need for identification of new therapies. In contrast to overconsumption of calories, epidemiological and experimental data has long supported restriction of calories as a preventative intervention against numerous forms of cancer.

Caloric restriction (CR) has historically been associated with what is known as continuous caloric restriction. Achieving continuous caloric restriction requires reducing macronutrient content daily, while remaining nutritionally sufficient. While initial studies investigated efficacy in prolonging life as well to prevent/delay tumor progression in animal models. Subsequent studies have investigated the percentage of CR as well as the macronutrient distribution of restriction. However, despite success in animal models, translational success of such interventions has been limited, in part due to the extreme difficulty in adhering to such rigorous dietary strategies long-term. Therefore, alternative strategies that result in restriction of energy intake in a noncontiguous manner have become an important evolution of this promising intervention.

Unlike continuous energy restriction attributed to CR, Intermittent fasting (IF) is restricting caloric intake in a noncontiguous manner ranging from fasting every other day up to a week at a time; it too holds promise as a cancer deterrent.

Chrononutrition, a form of IF which has received recent investigational interest, elucidates the interaction between eating and circadian rhythms and how these interactions affect biological functions such as metabolism. Current public health research tells us disturbing circadian rhythms (i.e. late night eating, night work shifts, insomnia, etc.) contributes not only to obesity (289, 290), but to liver cancer as well (291). One approach to fasting that considers circadian rhythms is time-restricted feeding, an eating pattern which restricts food intake to a specific period of time, which has demonstrated promise in combating obesity in animal models. To date, no evidence exists demonstrating the efficacy of time-restricted feeding in liver cancer development in the setting of circadian rhythm dysfunction.

For many nutrition health experts, the goal in curbing obesity related diseases is to identify obtainable and sustainable lifestyle interventions. However, dietary or lifestyle interventions have largely been shown to be ineffective due to lack of adherence (292). Time-restricted feeding offers clinical promise because it is simple (requiring little to no expertise for execution), obtainable (requiring minimal lifestyle interventions) and sustainable (does not alter diet composition) over the long-term.

In addition to identifying dietary strategies used to deter HCC, elucidating the molecular mechanisms that drive hepatic cancer are important events to be studied. Despite the clear relationship obesity and NAFLD have with cancer, the mechanisms underlying this relationship have not been fully elucidated. In the past decade, the field of cancer biology has seen an explosion of research into

the area of energy metabolism. A major emphasis of these studies has been on glucose and glutamine utilization and lipogenesis (64, 86, 93, 293), pathways that are robustly altered in cancer cells and that are largely conserved across different types of cancer. In addition, research on anabolic signaling pathways (Akt, mTOR, Ras/Raf, etc.) that are activated in response to excess calories and that are also increased in cancer cells have also been a major research focus. Catabolic metabolism has almost exclusively focused on glucose and amino acid metabolism. Lipolysis in cancer on the other hand, has primarily been studied in the context of cachexia (225, 294), leaving a dearth of knowledge in this area of metabolism. However, with the recent discoveries that growth factors suppress ATGL (154) and that catecholamine induced lipolysis ablates mTORC1 signaling in adipose tissue (227), there is a renewed focus on oncogenic metabolism. Thus, with anabolic signaling implicated in the pathophysiology of oncogenic events and development of liver cancer (228, 259, 295, 296), further investigation is warranted in identifying the molecular mechanism underlying ATGL and lipolysis in liver cancer.

We have recently shown that ATGL expression is driven by FoxO proteins, which largely drive pathways antagonistic to mTOR (220). Our laboratory has also shown that ATGL induces a signaling network that promotes FA oxidative metabolism and mitochondrial biogenesis while attenuating glucose utilization (109, 297). Further interrogation revealed that ATGL promotes these pathways thought its induction of the protein deacetylase SIRT1, which in turn regulates a downstream transcriptional network including PPAR-α, PGC-1α and

FoxO proteins known to govern oxidative metabolism, mitochondrial function and promote oxidative stress resistance (224). This transcriptional network, especially FoxO proteins, is known to have anti-proliferative effects (228, 298–300). Thus, future studies will want to explore the mechanistic link between ATGL as a mediator in tumor suppression as well as its role in explaining the benefits described from dietary interventions such as caloric restriction or time restricted feeding. Such results will help to extrapolate causal links in dietary intervention studies as well as providing new therapeutic avenues in the prevention and treatment of liver cancer.

In summary, fasting metabolism perpetuated through dietary intervention or modulation of ATGL demonstrates significant promise in curbing a rapidly growing and deadly form of cancer in developed countries such as the United States. Future research will be needed to identify dietary and lifestyle interventions that are sustainable if we are to combat obesity related pathologies such as HCC. Deeper insight into the mechanism that drive these alterations will provide crucial insight that will help identify key molecular signatures of an otherwise largely understudied etiology of liver cancer. Information gained from such studies may help to provide new clinical tools through lifestyle modification or therapeutics.

Bibliography

1. Organization, I. A. for R. on C. W. H. 2013. World Health Organization Biennial Report: Section of Cancer Information (CIN). World Health Organization, Lyon, France.
2. El–Serag, H. B., and K. L. Rudolph. 2007. Hepatocellular Carcinoma: Epidemiology and Molecular Carcinogenesis. *Gastroenterology*. **132**: 2557–2576.
3. Dhanasekaran, R., A. Limaye, and R. Cabrera. 2012. Hepatocellular carcinoma: current trends in worldwide epidemiology, risk factors, diagnosis, and therapeutics. *Hepat. Med.* **4**: 19–37.
4. Surveillance Research Program, National Cancer Institute. Fast stats: an interactive tool for access to SEER cancer statistics. **2014**. [online] http://seer.cancer.gov/faststats.
5. Kastl, L., S. W. Sauer, T. Ruppert, T. Beissbarth, M. S. Becker, D. Suss, P. H. Krammer, and K. Gulow. 2014. TNF-alpha mediates mitochondrial uncoupling and enhances ROS-dependent cell migration via NF-kappaB activation in liver cells. *FEBS Lett.* **588**: 175–183.
6. Liu, F., X. You, X. Chi, T. Wang, L. Ye, J. Niu, and X. Zhang. 2014. Hepatitis B virus X protein mutant HBxDelta127 promotes proliferation of hepatoma cells through up-regulating miR-215 targeting PTPRT. *Biochem. Biophys. Res. Commun.*
7. Liu, Y., and F. Wu. 2010. Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environ. Health Perspect.* **118**: 818–824.
8. McMahon, B. J., L. R. Bulkow, R. J. Singleton, J. Williams, M. Snowball, C. Homan, and A. J. Parkinson. 2011. Elimination of hepatocellular carcinoma and acute hepatitis B in children 25 years after a hepatitis B newborn and catch-up immunization program. *Hepatology*. **54**: 801–807.
9. El-Serag, H. B. 2002. Hepatocellular carcinoma and hepatitis C in the United States. *Hepatology*. **36**: S74-83.
10. Ludwig, J., T. Viggiano, D. McGill, and B. Oh. 1980. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc.* **55(7)**: 434–8.
11. Rosmorduc, O., and L. Fartoux. 2012. HCC and NASH: how strong is the clinical demonstration? *Clin. Res. Hepatol. Gastroenterol.* **36**: 202–208.
12. Bedogni, G., L. Miglioli, F. Masutti, C. Tiribelli, G. Marchesini, and S. Bellentani. 2005. Prevalence of and risk factors for nonalcoholic fatty liver disease: the Dionysos nutrition and liver study. *Hepatology*. **42**: 44–52.
13. Adams, L. A., J. F. Lymp, J. St Sauver, S. O. Sanderson, K. D. Lindor, A. Feldstein, and P. Angulo. 2005. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology*. **129**:

113–121.
14. Ascha, M. S., I. A. Hanouneh, R. Lopez, T. A. Tamimi, A. F. Feldstein, and N. N. Zein. 2010. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology*. **51**: 1972–1978.
15. Bugianesi, E., N. Leone, E. Vanni, G. Marchesini, F. Brunello, P. Carucci, A. Musso, P. De Paolis, L. Capussotti, M. Salizzoni, and M. Rizzetto. 2002. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology*. **123**: 134–140.
16. Powell, E. E., W. G. Cooksley, R. Hanson, J. Searle, J. W. Halliday, and L. W. Powell. 1990. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology*. **11**: 74–80.
17. Baffy, G., E. M. Brunt, and S. H. Caldwell. 2012. Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. *J. Hepatol.* **56**: 1384–1391.
18. Cuadrado, A., A. Orive, C. Garcia-Suarez, A. Dominguez, J. C. Fernandez-Escalante, J. Crespo, and F. Pons-Romero. 2005. Non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma. *Obes. Surg.* **15**: 442–446.
19. Kawada, N., K. Imanaka, T. Kawaguchi, C. Tamai, R. Ishihara, T. Matsunaga, K. Gotoh, T. Yamada, and Y. Tomita. 2009. Hepatocellular carcinoma arising from non-cirrhotic nonalcoholic steatohepatitis. *J. Gastroenterol.* **44**: 1190–1194.
20. Shrager, B., G. Jibara, M. Schwartz, and S. Roayaie. 2012. Resection of hepatocellular carcinoma without cirrhosis. *Ann. Surg.* **255**: 1135–1143.
21. Nagasue, N., H. Kohno, Y. C. Chang, H. Taniura, A. Yamanoi, M. Uchida, T. Kimoto, Y. Takemoto, T. Nakamura, and H. Yukaya. 1993. Liver resection for hepatocellular carcinoma. Results of 229 consecutive patients during 11 years. *Ann. Surg.* **217**: 375–384.
22. Hui, J. M., J. G. Kench, S. Chitturi, A. Sud, G. C. Farrell, K. Byth, P. Hall, M. Khan, and J. George. 2003. Long-term outcomes of cirrhosis in nonalcoholic steatohepatitis compared with hepatitis C. *Hepatology*. **38**: 420–427.
23. Marrero, J. A., R. J. Fontana, G. L. Su, H. S. Conjeevaram, D. M. Emick, and A. S. Lok. 2002. NAFLD may be a common underlying liver disease in patients with hepatocellular carcinoma in the United States. *Hepatology*. **36**: 1349–1354.
24. Obesity and Overweight. 2016. 1. [online] http://www.who.int/mediacentre/factsheets/fs311/en/ (Accessed April 6,

2017).
25. Flegal, K. M., M. D. Carroll, B. K. Kit, and C. L. Ogden. 2012. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *JAMA*. **307**: 491–497.
26. Ebbeling, C. B., D. B. Pawlak, and D. S. Ludwig. 2002. Childhood obesity: public-health crisis, common sense cure. *Lancet*. **360**: 473–482.
27. Wolin, K., K. Carson, and G. Colditz. 2010. Obesity and cancer. *Oncologist*. **15**: 556–565.
28. Wolk, A., G. Gridley, M. Svensson, O. Nyren, J. K. McLaughlin, J. F. Fraumeni, and H. O. Adam. 2001. A prospective study of obesity and cancer risk (Sweden). *Cancer Causes Control*. **12**: 13–21.
29. Calle, E. E., C. Rodriguez, K. Walker-Thurmond, and M. J. Thun. 2003. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N. Engl. J. Med.* **348**: 1625–1638.
30. Vanni, E., and E. Bugianesi. 2014. Obesity and liver cancer. *Clin. Liver Dis.* **18**: 191–203.
31. Saunders, D., D. Seidel, M. Allison, and G. Lyratzopoulos. 2010. Systematic review: the association between obesity and hepatocellular carcinoma - epidemiological evidence. *Aliment. Pharmacol. Ther.* **31**: 1051–1063.
32. Chang, T.-Y., and J.-D. Chen. 2012. Fatty Liver and Metabolic Syndrome in Nonabdominally Obese Taiwanese Adults. *Asia-Pacific J. Public Heal.* **24**: 472–479.
33. Petta, S., M. C. Amato, V. Di Marco, C. Camma, G. Pizzolanti, M. R. Barcellona, D. Cabibi, A. Galluzzo, D. Sinagra, C. Giordano, and A. Craxi. 2012. Visceral adiposity index is associated with significant fibrosis in patients with non-alcoholic fatty liver disease. *Aliment. Pharmacol. Ther.* **35**: 238–247.
34. Ohki, T., R. Tateishi, S. Shiina, E. Goto, T. Sato, H. Nakagawa, R. Masuzaki, T. Goto, K. Hamamura, F. Kanai, H. Yoshida, T. Kawabe, and M. Omata. 2009. Visceral fat accumulation is an independent risk factor for hepatocellular carcinoma recurrence after curative treatment in patients with suspected NASH. *Gut*. **58**: 839–844.
35. Schlesinger, S., K. Aleksandrova, T. Pischon, V. Fedirko, M. Jenab, E. Trepo, P. Boffetta, C. C. Dahm, K. Overvad, A. Tjonneland, J. Halkjaer, G. Fagherazzi, M.-C. Boutron-Ruault, F. Carbonnel, R. Kaaks, A. Lukanova, H. Boeing, A. Trichopoulou, C. Bamia, P. Lagiou, D. Palli, S. Grioni, S. Panico, R. Tumino, P. Vineis, H. B. Bueno-de-Mesquita, S. van den Berg, P. H. M. Peeters, T. Braaten, E. Weiderpass, J. R. Quiros, N. Travier, M.-J. Sanchez, C. Navarro, A. Barricarte, M. Dorronsoro, B. Lindkvist, S. Regner, M. Werner, M. Sund, K.-T. Khaw, N. Wareham, R. C. Travis, T.

Norat, P. A. Wark, E. Riboli, and U. Nothlings. 2013. Abdominal obesity, weight gain during adulthood and risk of liver and biliary tract cancer in a European cohort. *Int. J. Cancer*. **132**: 645–657.
36. Zhao, J., and M. W. Lawless. 2013. Stop feeding cancer: Pro-inflammatory role of visceral adiposity in liver cancer. *Cytokine*. **64**: 626–637.
37. Diabetes: Data and Statistics. 2017. [online] https://www.cdc.gov/diabetes/data/.
38. Prevention, C. for D. C. and. 2011. National Diabetes Fact Sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, GA.
39. Adami, H. O., W. H. Chow, O. Nyren, C. Berne, M. S. Linet, A. Ekbom, A. Wolk, J. K. McLaughlin, and J. F. Fraumeni Jr. 1996. Excess risk of primary liver cancer in patients with diabetes mellitus. *J. Natl. Cancer Inst.* **88**: 1472–1477.
40. Davila, J. A., R. O. Morgan, Y. Shaib, K. A. McGlynn, and H. B. El-Serag. 2005. Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. *Gut*. **54**: 533–539.
41. El-Serag, H. B., H. Hampel, and F. Javadi. 2006. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. *Clin. Gastroenterol. Hepatol.* **4**: 369–380.
42. Lai, M.-S., M.-S. Hsieh, Y.-H. Chiu, and T. H.-H. Chen. 2006. Type 2 diabetes and hepatocellular carcinoma: A cohort study in high prevalence area of hepatitis virus infection. *Hepatology*. **43**: 1295–1302.
43. Shintani, Y., H. Fujie, H. Miyoshi, T. Tsutsumi, K. Tsukamoto, S. Kimura, K. Moriya, and K. Koike. 2004. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology*. **126**: 840–848.
44. Loria, P., A. Lonardo, and F. Anania. 2013. Liver and diabetes. A vicious circle. *Hepatol. Res.* **43**: 51–64.
45. Day, C. P., and O. F. W. James. 1998. Steatohepatitis: A tale of two “hits”? *Gastroenterology*. **114**: 842–845.
46. Speliotes, E. K., J. M. Massaro, U. Hoffmann, R. S. Vasan, J. B. Meigs, D. V Sahani, J. N. Hirschhorn, C. J. O’Donnell, and C. S. Fox. 2010. Fatty Liver is Associated With Dyslipidemia and Dysglycemia Independent of Visceral Fat: The Framingham Heart Study. *Hepatology*. **51**: 1979–1987.
47. Bedogni, G., A. Gastaldelli, M. Manco, A. De Col, F. Agosti, C. Tiribelli, and A. Sartorio. 2012. Relationship between fatty liver and glucose metabolism: a cross-sectional study in 571 obese children. *Nutr Metab Cardiovasc Dis.* **22**: 120–6.

48. Wong, V. W. S., A. Y. Hui, S. W. C. Tsang, J. L. Y. Chan, G. L. H. Wong, A. W. H. Chan, W. Y. So, A. Y. S. Cheng, P. C. Y. Tong, F. K. L. Chan, J. J. Y. Sung, and H. L. Y. Chan. 2006. Prevalence of undiagnosed diabetes and postchallenge hyperglycaemia in Chinese patients with non-alcoholic fatty liver disease. *Aliment. Pharmacol. Ther.* **24**: 1215–1222.
49. Marchesini, G., M. Brizi, G. Bianchi, S. Tomassetti, E. Bugianesi, M. Lenzi, A. J. McCullough, S. Natale, G. Forlani, and N. Melchionda. 2001. Nonalcoholic fatty liver disease - A feature of the metabolic syndrome. *Diabetes*. **50**: 1844–1850.
50. Adams, L. A., O. R. Waters, M. W. Knuiman, R. R. Elliott, and J. K. Olynyk. 2009. NAFLD as a Risk Factor for the Development of Diabetes and the Metabolic Syndrome: An Eleven-Year Follow-up Study. *Am. J. Gastroenterol.* **104**: 861–867.
51. Balkau, B., C. Lange, S. Vol, F. Fumeron, F. Bonnet, and G. S. DESIR. 2010. Nine-year incident diabetes is predicted by fatty liver indices: the French DESIR study. *Bmc Gastroenterol.* **10**: 56.
52. Bae, J. C., E. J. Rhee, W. Y. Lee, S. E. Park, C. Y. Park, K. W. Oh, S. W. Park, and S. W. Kim. 2011. Combined Effect of Nonalcoholic Fatty Liver Disease and Impaired Fasting Glucose on the Development of Type 2 Diabetes A 4-year retrospective longitudinal study. *Diabetes Care*. **34**: 727–729.
53. Adams, L. A., S. Sanderson, K. D. Lindor, and P. Angulo. 2005. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J. Hepatol.* **42**: 132–138.
54. Paradis, V., S. Zalinski, E. Chelbi, N. Guedj, F. Degos, V. Vilgrain, P. Bedossa, and J. Belghiti. 2009. Hepatocellular Carcinomas in Patients With Metabolic Syndrome Often Develop Without Significant Liver Fibrosis: A Pathological Analysis. *Hepatology*. **49**: 851–859.
55. Ertle, J., A. Dechene, J.-P. FAU - Sowa, S. JP, V. FAU - Penndorf, V. Penndorf, K. FAU - Herzer, K. Herzer, G. FAU - Kaiser, G. Kaiser, J. F. FAU - Schlaak, S. JF, G. FAU - Gerken, G. Gerken, W.-K. FAU - Syn, S. WK, A. FAU - Canbay, and A. Canbay. 2011. Non-alcoholic fatty liver disease progresses to hepatocellular carcinoma in the absence of apparent cirrhosis. *Int J Cancer.* **128**: 2436–43.
56. Warburg, O., F. Wind, and E. Negelein. 1927. The Metabolism of Tumors in the Body. *J Gen Physiol.* **8**: 519–30.
57. Swerdlow, R. H., E. Lezi, D. Aires, and J. Lu. 2013. Glycolysis-respiration relationships in a neuroblastoma cell line. *Biochim. Biophys. Acta-General Subj.* **1830**: 2891–2898.
58. Warburg, O. 1956. On the Origin of Cancer Cells. *Science (80-.).* **123**: 309–

314.
59. Fantin, V., J. St-Pierre, and P. Leder. 2006. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell*. **9(6)**: 425–34.
60. Metallo, C., P. Gameiro, E. Bell, K. Mattaini, J. Yang, K. Hiller, C. Jewel, Z. Johnson, D. Irvine, L. Guarente, J. Kelleher, M. Vander Heiden, O. Iliopoulos, and G. Stephanopoulos. 2011. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature*. **481**: 380–4.
61. Hara, K., K. Yonezawa, Q. P. Weng, M. T. Kozlowski, C. Belham, and J. Avruch. 1998. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J. Biol. Chem.* **273**: 14484–14494.
62. Krause, U., L. Bertrand, L. Maisin, M. Rosa, and L. Hue. 2002. Signalling pathways and combinatory effects of insulin and amino acids in isolated rat hepatocytes. *Eur. J. Biochem.* **269**: 3742–3750.
63. Carpenter, G., and S. Cohen. 1979. Epidermal growth factor. - *Annu Rev Biochem.* **48**: 193–216.
64. DeBerardinis, R. J., J. J. Lum, G. Hatzivassiliou, and C. B. Thompson. 2008. The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. *Cell Metab.* **7**: 11–20.
65. Cappuzzo, F., H. FR, E. Rossi, S. Bartolini, C. GL, L. Bemis, J. Haney, S. Witta, K. Danenberg, I. Domenichini, V. Ludovini, E. Magrini, V. Gregorc, C. Doglioni, A. Sidoni, M. Tonato, F. WA, L. Crino, B. P. A. Jr, and M. Varella-Garcia. 2005. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst.* **97(9)**: 643–55.
66. Jiang, W., Z. Zhu, and H. J. Thompson. 2013. Effects of limiting energy availability via diet and physical activity on mammalian target of rapamycin-related signaling in rat mammary carcinomas. *Carcinogenesis*. **34**: 378–87.
67. Moore, T., L. Beltran, S. Carbajal, S. Strom, J. Traag, S. D. Hursting, and J. DiGiovanni. 2008. Dietary Energy Balance Modulates Signaling through the Akt/Mammalian Target of Rapamycin Pathways in Multiple Epithelial Tissues. *Cancer Prev. Res.* **1**: 65–76.
68. Moore, T., S. Carbajal, L. Beltran, S. N. Perkins, S. Yakar, D. LeRoith, S. D. Hursting, and J. DiGiovanni. 2008. Reduced susceptibility to two-stage skin carcinogenesis in mice with low circulating insulin-like growth factor I levels. *Cancer Res.* **68**: 3680–3688.
69. Vander Heiden, M., N. Chandel, P. Schumacker, and C. Thompson. 1999. Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. *Mol Cell.* **3**: 159–67.

70. Kilburn, D., M. Lilly, and F. Webb. 1969. The energetics of mammalian cell growth. *J Cell Sci.* **4**: 645–54.
71. Christofk, H., M. Vander Heiden, M. Harris, A. Ramanathan, R. Gerszten, R. Wei, M. D. Fleming, S. Schreiber, and L. C. Cantley. 2008. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature.* **452**: 230–3.
72. Fang, Y., J.-L. Xue, Q. Shen, J. Chen, and L. Tian. 2012. MicroRNA-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in hepatocellular carcinoma. *Hepatology*. **55**: 1852–1862.
73. Codina, M., D. Garcia de la serrana, J. Sanchez-Gurmaches, N. Montserrat, O. Chistyakova, I. Navarro, and J. Gutierrez. 2008. Metabolic and mitogenic effects of IGF-II in rainbow trout (Oncorhynchus mykiss) myocytes in culture and the role of IGF-II in the PI3K/Akt and MAPK signalling pathways. *Gen. Comp. Endocrinol.* **157**: 116–124.
74. Sun, Q., X. Chen, J. Ma, H. Peng, F. Wang, X. Zha, Y. Wang, Y. Jing, H. Yang, R. Chen, L. Chang, Y. Zhang, J. Goto, H. Onda, T. Chen, M.-R. Wang, Y. Lu, H. You, D. Kwiatkowski, and H. Zhang. 2011. Mammalian target of rapamycin up-regulation of pyruvate kinase isoenzyme type M2 is critical for aerobic glycolysis and tumor growth. *Proc. Natl. Acad. Sci. U. S. A.* **108**: 4129–4134.
75. Cordero-Espinoza, L., and T. Hagen. 2013. Increased Concentrations of Fructose 2,6-Bisphosphate Contribute to the Warburg Effect in Phosphatase and Tensin Homolog (PTEN)-deficient Cells. *J. Biol. Chem.* **288**: 36020–36028.
76. Koolman, J., and K. H. Roehn. 2005. Color Atlas of Biochemistry. Thieme, Stuttgart, Germany.
77. Jurica, M. S., A. Mesecar, P. J. Heath, W. X. Shi, T. Nowak, and B. L. Stoddard. 1998. The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. *Structure*. **6**: 195–210.
78. Christofk, H. R., M. G. Vander Heiden, N. Wu, J. M. Asara, and L. C. Cantley. 2008. Pyruvate kinase M2 is a phosphotyrosine-binding protein. *Nature*. **452**: 181-U27.
79. Liu, A. M., Z. Xu, F. H. Shek, K.-F. Wong, N. P. Lee, R. T. Poon, J. Chen, and J. M. Luk. 2014. miR-122 Targets Pyruvate Kinase M2 and Affects Metabolism of Hepatocellular Carcinoma. *PLoS One*. **9**: e86872–e86872.
80. Birsoy, K., R. Possemato, F. K. Lorbeer, E. C. Bayraktar, P. Thiru, B. Yucel, T. Wang, W. W. Chen, C. B. Clish, and D. M. Sabatini. 2014. Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. *Nature*. **508**: 108–12.

81. Wu, G. 2009. Amino acids: metabolism, functions, and nutrition. *Amino Acids*. **37**: 1–17.
82. Eagle, H. 1955. Nutrition Needs of Mammalian Cells in Tissue Culture. *Science (80-.).* **122**: 501–504.
83. Le, A., A. N. Lane, M. Hamaker, S. Bose, A. Gouw, J. Barbi, T. Tsukamoto, C. J. Rojas, B. S. Slusher, H. Zhang, L. J. Zimmerman, D. C. Liebler, R. J. C. Slebos, P. K. Lorkiewicz, R. M. Higashi, T. W. M. Fan, and C. V Dang. 2012. Glucose-Independent Glutamine Metabolism via TCA Cycling for Proliferation and Survival in B Cells. *Cell Metab.* **15**: 110–121.
84. Martin-Rufian, M., R. Nascimento-Gomes, A. Higuero, A. R. Crisma, J. A. Campos-Sandoval, M. C. Gomez-Garcia, C. Cardona, T. Cheng, C. Lobo, J. A. Segura, F. J. Alonso, M. Szeliga, J. Albrecht, R. Curi, J. Marquez, A. Colquhoun, R. J. DeBerardinis, and J. M. Mates. 2014. Both GLS silencing and GLS2 overexpression synergize with oxidative stress against proliferation of glioma cells. *J. Mol. Med.* **92**: 277–290.
85. DeBerardinis, R. J., and T. Cheng. 2010. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene*. **29**: 313–324.
86. DeBerardinis, R. J., N. Sayed, D. Ditsworth, and C. B. Thompson. 2008. Brick by brick: metabolism and tumor cell growth. *Curr. Opin. Genet. Dev.* **18**: 54–61.
87. Gao, P., I. Tchernyshyov, T.-C. Chang, Y.-S. Lee, K. Kita, T. Ochi, K. I. Zeller, A. M. De Marzo, J. E. Van Eyk, J. T. Mendell, and C. V Dang. 2009. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature*. **458**: 762-U100.
88. Tessem, M.-B., M. G. Swanson, K. R. Keshari, M. J. Albers, D. Joun, Z. L. Tabatabai, J. P. Simko, K. Shinohara, S. J. Nelson, D. B. Vigneron, I. S. Gribbestad, and J. Kurhanewicz. 2008. Evaluation of lactate and alanine as metabolic biomarkers of prostate cancer using H-1 HR-MAS spectroscopy of biopsy tissues. *Magn. Reson. Med.* **60**: 510–516.
89. DeBerardinis, R., A. Mancuso, I. Daikhin, ENissim, M. Yudkoff, S. Wehrli, and C. B. Thompson. 2007. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci USA.* **104**: 19345–50.
90. Meng, T. C., T. Fukada, and N. K. Tonks. 2002. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol. Cell*. **9**: 387–399.
91. David, S. S., V. L. O'Shea, and S. Kundu. 2007. Base-excision repair of oxidative DNA damage. *Nature*. **447**: 941–950.
92. Lo, M., Y.-Z. Wang, and P. W. Gout. 2008. The x(c)(-) cystine/glutamate antiporter: A potential target for therapy of cancer and other diseases. *J. Cell. Physiol.* **215**: 593–602.

93. Wise, D. R., and C. B. Thompson. 2010. Glutamine addiction: a new therapeutic target in cancer. *Trends Biochem. Sci.* **35**: 427–433.
94. Nicklin, P., P. Bergman, B. Zhang, E. Triantafellow, H. Wang, B. Nyfeler, H. Yang, M. Hild, C. Kung, C. Wilson, V. E. Myer, J. P. MacKeigan, J. A. Porter, Y. K. Wang, L. C. Cantley, P. M. Finan, and L. O. Murphy. 2009. Bidirectional Transport of Amino Acids Regulates mTOR and Autophagy. *Cell*. **136**: 521–534.
95. Singh, R., and A. M. Cuervo. 2011. Autophagy in the Cellular Energetic Balance. *Cell Metab.* **13**: 495–504.
96. Dang, C. V. 2012. Links between metabolism and cancer. *Genes Dev.* **26**: 877–890.
97. Wellen, K. E., and C. B. Thompson. 2010. Cellular Metabolic Stress: Considering How Cells Respond to Nutrient Excess. *Mol. Cell*. **40**: 323–332.
98. Ookhtens, M., R. Kannan, I. Lyon, and N. Baker. 1984. Liver and adipose tissue contributions to newly formed fatty acids in an ascites tumor. *Am J Physiol.* **247**: R146-53.
99. Timlin, M. T., and E. J. Parks. 2005. Temporal pattern of de novo lipogenesis in the postprandial state in healthy men. *Am. J. Clin. Nutr.* **81**: 35–42.
100. Donnelly, K. L., C. I. Smith, S. J. Schwarzenberg, J. Jessurun, M. D. Boldt, and E. J. Parks. 2005. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J. Clin. Invest.* **115**: 1343–1351.
101. Kannan, R., L. Irving, and N. Baker. 1980. Dietary Control of Lipogenesis in Vivo in Host Tissues and Tumors of MIce Bearing Ehrlich Ascites Carcinoma. *Cancer Res.* **40**: 4606–46011.
102. Black, P. N., and C. C. DiRusso. 2007. Vectorial acylation: linking fatty acid transport and activation to metabolic trafficking. *Novartis Found. Symp.* **286**: 127-141-163-203.
103. Bu, S. Y., and D. G. Mashek. 2010. Hepatic long-chain acyl-CoA synthetase 5 mediates fatty acid channeling between anabolic and catabolic pathways. *J. Lipid Res.* **51**: 3270–3280.
104. Fujimoto, Y., H. Itabe, T. Kinoshita, K. J. Homma, J. Onoduka, M. Mori, S. Yamaguchi, M. Makita, Y. Higashi, A. Yamashita, and T. Takano. 2007. Involvement of ACSL in local synthesis of neutral lipids in cytoplasmic lipid droplets in human hepatocyte HuH7. *J. Lipid Res.* **48**: 1280–1292.
105. Harada, N., Z. Oda, Y. Hara, K. Fujinami, M. Okawa, K. Ohbuchi, M. Yonemoto, Y. Ikeda, K. Ohwaki, K. Aragane, Y. Tamai, and J. Kusunoki. 2007. Hepatic de novo lipogenesis is present in liver-specific ACC1-deficient mice. *Mol. Cell. Biol.* **27**: 1881–1888.

106. Aarsland, A., and R. R. Wolfe. 1998. Hepatic secretion of VLDL fatty acids during stimulated lipogenesis in men. *J. Lipid Res.* **39**: 1280–1286.
107. Green, C. D., C. G. Ozguden-Akkoc, Y. Wang, D. B. Jump, and L. K. Olson. 2010. Role of fatty acid elongases in determination of de novo synthesized monounsaturated fatty acid species. *J. Lipid Res.* **51**: 1871–1877.
108. Cui, M., Y. Wang, B. Sun, Z. Xiao, L. Ye, and X. Zhang. 2014. MiR-205 modulates abnormal lipid metabolism of hepatoma cells via targeting acyl-CoA synthetase long-chain family member 1 (ACSL1) mRNA. *Biochem. Biophys. Res. Commun.* **444**: 270–275.
109. Ong, K. T., M. T. Mashek, S. Y. Bu, A. S. Greenberg, and D. G. Mashek. 2011. Adipose triglyceride lipase is a major hepatic lipase that regulates triacylglycerol turnover and fatty acid signaling and partitioning. *Hepatology*. **53**: 116–126.
110. Kleiner, D. E., P. D. Berk, J. Y. Hsu, A. P. Courcoulas, D. Flum, S. Khandelwal, J. Pender, A. Pomp, J. Roerig, L. L. Machado, B. M. Wolfe, S. H. Belle, and L. Consortium. 2014. Hepatic pathology among patients without known liver disease undergoing bariatric surgery: observations and a perspective from the longitudinal assessment of bariatric surgery (LABS) study. *Semin. Liver Dis.* **34**: 98–107.
111. Wendel, A. A., D. E. Cooper, O. R. Ilkayeva, D. M. Muoio, and R. A. Coleman. 2013. Glycerol-3-phosphate acyltransferase (GPAT)-1, but not GPAT4, incorporates newly synthesized fatty acids into triacylglycerol and diminishes fatty acid oxidation. *J. Biol. Chem.* **288**: 27299–27306.
112. Ellis, J. M., D. S. Paul, M. A. DePetrillo, B. P. Singh, D. E. Malarkey, and R. A. Coleman. 2012. Mice Deficient in Glycerol-3-Phosphate Acyltransferase-1 Have a Reduced Susceptibility to Liver Cancer. *Toxicol. Pathol.* **40**: 513–521.
113. Monetti, M., M. C. Levin, M. J. Watt, M. P. Sajan, S. Marmor, B. K. Hubbard, R. D. Stevens, J. R. Bain, C. B. Newgard, S. Farese RV, A. L. Hevener, and R. V Farese Jr. 2007. Dissociation of hepatic steatosis and insulin resistance in mice overexpressing DGAT in the liver. *Cell Metab.* **6**: 69–78.
114. Lee, K., J.-I. Goo, H. Y. Jung, M. Kim, S. K. Boovanahalli, H. R. Park, M.-O. Kim, D.-H. Kim, H. S. Lee, and Y. Choi. 2012. Discovery of a novel series of benzimidazole derivatives as diacylglycerol acyltransferase inhibitors. *Bioorg. Med. Chem. Lett.* **22**: 7456–7460.
115. Hall, A. M., K. Kou, Z. Chen, T. A. Pietka, M. Kumar, K. M. Korenblat, K. Lee, K. Ahn, E. Fabbrini, S. Klein, B. Goodwin, and B. N. Finck. 2012. Evidence for regulated monoacylglycerol acyltransferase expression and activity in human liver. *J. Lipid Res.* **53**: 990–999.
116. Sookoian, S., G. O. Castano, A. L. Burgueno, T. Fernandez Gianotti, M.

Soledad Rosselli, and C. Jose Pirola. 2009. A nonsynonymous gene variant in the adiponutrin gene is associated with nonalcoholic fatty liver disease severity. *J. Lipid Res.* **50**: 2111–2116.

117. Hassan, M. M., A. Kaseb, C. J. Etzel, H. El-Serag, M. R. Spitz, P. Chang, K. S. Hale, M. Liu, A. Rashid, M. Shama, J. L. Abbruzzese, E. M. Loyer, H. Kaur, H. M. Hassabo, J.-N. Vauthey, C. J. Wray, B. S. Hassan, Y. Z. Patt, E. Hawk, K. M. Soliman, and D. Li. 2013. Genetic Variation in the PNPLA3 Gene and Hepatocellular Carcinoma in USA: Risk and Prognosis Prediction. *Mol. Carcinog.* **52**: 139–147.
118. Singal, A. G., H. Manjunath, A. C. Yopp, M. S. Beg, J. A. Marrero, P. Gopal, and A. K. Waljee. 2014. The Effect of PNPLA3 on Fibrosis Progression and Development of Hepatocellular Carcinoma: A Meta-analysis. *Am. J. Gastroenterol.* **109**: 325–334.
119. He, S., C. McPhaul, J. Z. Li, R. Garuti, L. Kinch, N. V Grishin, J. C. Cohen, and H. H. Hobbs. 2010. A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. *J. Biol. Chem.* **285**: 6706–6715.
120. Kumari, M., G. Schoiswohl, C. Chitraju, M. Paar, I. Cornaciu, A. Y. Rangrez, N. Wongsiriroj, H. M. Nagy, P. T. Ivanova, S. A. Scott, O. Knittelfelder, G. N. Rechberger, R. Birner-Gruenberger, S. Eder, H. A. Brown, G. Haemmerle, M. Oberer, A. Lass, E. E. Kershaw, R. Zimmermann, and R. Zechner. 2012. Adiponutrin Functions as a Nutritionally Regulated Lysophosphatidic Acid Acyltransferase. *Cell Metab.* **15**: 691–702.
121. Ruhanen, H., J. Perttila, M. Holtta-Vuori, Y. Zhou, H. Yki-Jarvinen, E. Ikonen, R. Kakela, and V. M. Olkkonen. 2014. PNPLA3 mediates hepatocyte triacylglycerol remodeling. *J. Lipid Res.* **55**: 739–746.
122. Le Bacquer, O., E. Petroulakis, S. Paglialunga, F. Poulin, D. Richard, K. Cianflone, and N. Sonenberg. 2007. Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2. *J. Clin. Invest.* **117**: 387–396.
123. Zhang, H. H., J. Huang, K. Duevel, B. Boback, S. Wu, R. M. Squillace, C.-L. Wu, and B. D. Manning. 2009. Insulin Stimulates Adipogenesis through the Akt-TSC2-mTORC1 Pathway. *PLoS One*. **4**: e6189–e6189.
124. Akram, M. 2013. A Focused Review of the Role of Ketone Bodies in Health and Disease. *J. Med. Food*. **16**: 965–967.
125. Sengupta, S., T. R. Peterson, M. Laplante, S. Oh, and D. M. Sabatini. 2010. mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. *Nature*. **468**: 1100-U502.
126. Kim, K., S. Pyo, and S. H. Um. 2012. S6 kinase 2 deficiency enhances ketone body production and increases peroxisome proliferator-activated

receptor alpha activity in the liver. *Hepatology*. **55**: 1727–1737.
127. Chakrabarti, P., J. Y. Kim, M. Singh, Y.-K. Shin, J. Kim, J. Kumbrink, Y. Wu, M.-J. Lee, K. H. Kirsch, S. K. Fried, and K. V Kandror. 2013. Insulin Inhibits Lipolysis in Adipocytes via the Evolutionarily Conserved mTORC1-Egr1-ATGL-Mediated Pathway. *Mol. Cell. Biol.* **33**: 3659–3666.
128. Porstmann, T., C. R. Santos, B. Griffiths, M. Cully, M. Wu, S. Leevers, J. R. Griffiths, Y. L. Chung, and A. Schulze. 2008. SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. *Cell Metab.* **8**: 224–236.
129. Duvel, K., J. L. Yecies, S. Menon, P. Raman, A. I. Lipovsky, A. L. Souza, E. Triantafellow, Q. Ma, R. Gorski, S. Cleaver, M. G. Vander Heiden, J. P. MacKeigan, P. M. Finan, C. B. Clish, L. O. Murphy, and B. D. Manning. 2010. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol. Cell*. **39**: 171–183.
130. Calvisi, D. F., C. Wang, C. Ho, S. Ladu, S. A. Lee, S. Mattu, G. Destefanis, S. Delogu, A. Zimmermann, J. Ericsson, S. Brozzetti, T. Staniscia, X. Chen, F. Dombrowski, and M. Evert. 2011. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. *Gastroenterology*. **140**: 1071–1083.
131. Owen, J. L., Y. Zhang, S. H. Bae, M. S. Farooqi, G. Liang, R. E. Hammer, J. L. Goldstein, and M. S. Brown. 2012. Insulin stimulation of SREBP-1c processing in transgenic rat hepatocytes requires p70 S6-kinase. *Proc. Natl. Acad. Sci. U. S. A.* **109**: 16184–16189.
132. Peterson, T. R., S. S. Sengupta, T. E. Harris, A. E. Carmack, S. A. Kang, E. Balderas, D. A. Guertin, K. L. Madden, A. E. Carpenter, B. N. Finck, and D. M. Sabatini. 2011. mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. *Cell*. **146**: 408–420.
133. Yecies, J. L., H. H. Zhang, S. Menon, S. Liu, D. Yecies, A. I. Lipovsky, C. Gorgun, D. J. Kwiatkowski, G. S. Hotamisligil, C. H. Lee, and B. D. Manning. 2011. Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. *Cell Metab.* **14**: 21–32.
134. Hagiwara, A., M. Cornu, N. Cybulski, P. Polak, C. Betz, F. Trapani, L. Terracciano, M. H. Heim, M. A. Rueegg, and M. N. Hall. 2012. Hepatic mTORC2 Activates Glycolysis and Lipogenesis through Akt, Glucokinase, and SREBP1c. *Cell Metab.* **15**: 725–738.
135. Li, C., W. Yang, J. Zhang, X. Zheng, Y. Yao, K. Tu, and Q. Liu. 2014. SREBP-1 Has a Prognostic Role and Contributes to Invasion and Metastasis in Human Hepatocellular Carcinoma. *Int. J. Mol. Sci.* **15**: 7124–7138.

136. Medes, G., A. Thomas, and S. Weinhouse. 1953. Metabolism of neoplastic tissue. IV. A study of lipid synthesis in neoplastic tissue slices in vitro. *Cancer Res.* **13**: 27–29.
137. Hatzivassiliou, G., F. P. Zhao, D. E. Bauer, C. Andreadis, A. N. Shaw, D. Dhanak, S. R. Hingorani, D. A. Tuveson, and C. B. Thompson. 2005. ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer Cell*. **8**: 311–321.
138. Yahagi, N., H. Shimano, K. Hasegawa, K. Ohashi, T. Matsuzaka, Y. Najima, M. Sekiya, S. Tomita, H. Okazaki, Y. Tamura, Y. Iizuka, K. Ohashi, R. Nagai, S. Ishibashi, T. Kadowaki, M. Makuuchi, S. Ohnishi, J. Osuga, and N. Yamada. 2005. Co-ordinate activation of lipogenic enzymes in hepatocellular carcinoma. *Eur. J. Cancer*. **41**: 1316–1322.
139. Brusselmans, K., E. De Schrijver, G. Verhoeven, and J. V Swinnen. 2005. RNA interference-mediated silencing of the Acetyl-CoA-Carboxylase-alpha gene induces growth inhibition and apoptosis of prostate cancer cells. *Cancer Res.* **65**: 6719–6725.
140. Poteet, E., G. R. Choudhury, A. Winters, W. Li, M.-G. Ryou, R. Liu, L. Tang, A. Ghorpade, Y. Wen, F. Yuan, S. T. Keir, H. Yan, D. D. Bigner, J. W. Simpkins, and S.-H. Yang. 2013. Reversing the Warburg Effect as a Treatment for Glioblastoma. *J. Biol. Chem.* **288**: 9153–9164.
141. Jeon, S.-M., N. S. Chandel, and N. Hay. 2012. AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. *Nature*. **485**: 661–+.
142. Jump, D. B., M. Torres-Gonzalez, and L. K. Olson. 2011. Soraphen A, an inhibitor of acetyl CoA carboxylase activity, interferes with fatty acid elongation. *Biochem. Pharmacol.* **81**: 649–660.
143. Menendez, J., and R. Lupu. 2007. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer.* **7**: 763–77.
144. Yang, Y. A., P. J. Morin, W. F. Han, T. H. Chen, D. M. Bornman, E. W. Gabrielson, and E. S. Pizer. 2003. Regulation of fatty acid synthase expression in breast cancer by sterol regulatory, element binding protein-1c. *Exp. Cell Res.* **282**: 132–137.
145. Turrado, C., T. Puig, J. Garcia-Carceles, M. Artola, B. Benhamu, S. Ortega-Gutierrez, J. Relat, G. Oliveras, A. Blancafort, D. Haro, P. F. Marrero, R. Colomer, and M. L. Lopez-Rodriguez. 2012. New Synthetic Inhibitors of Fatty Acid Synthase with Anticancer Activity. *J. Med. Chem.* **55**: 5013–5023.
146. Matsuzaka, T., A. Atsumi, R. Matsumori, T. Nie, H. Shinozaki, N. Suzuki-Kemuriyama, M. Kuba, Y. Nakagawa, K. Ishii, M. Shimada, K. Kobayashi, S. Yatoh, A. Takahashi, K. Takekoshi, H. Sone, N. Yahagi, H. Suzuki, S.

Murata, M. Nakamuta, N. Yamada, and H. Shimano. 2012. Elovl6 Promotes Nonalcoholic Steatohepatitis. *Hepatology*. **56**: 2199–2208.
147. Roongta, U. V, J. G. Pabalan, X. Wang, R.-P. Ryseck, J. Fargnoli, B. J. Henley, W.-P. Yang, J. Zhu, M. T. Madireddi, R. M. Lawrence, T. W. Wong, and B. A. Rupnow. 2011. Cancer Cell Dependence on Unsaturated Fatty Acids Implicates Stearoyl-CoA Desaturase as a Target for Cancer Therapy. *Mol. Cancer Res.* **9**: 1551–1561.
148. Cao, Y., A. T. Pearman, G. A. Zimmerman, T. M. McIntyre, and S. M. Prescott. 2000. Intracellular unesterified arachidonic acid signals apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* **97**: 11280–11285.
149. Orlando, U. D., J. Garona, G. V Ripoll, P. M. Maloberti, A. R. Solano, A. Avagnina, D. E. Gomez, D. F. Alonso, and E. J. Podesta. 2012. The Functional Interaction between Acyl-CoA Synthetase 4, 5-Lipooxygenase and Cyclooxygenase-2 Controls Tumor Growth: A Novel Therapeutic Target. *PLoS One*. **7**: e40794–e40794.
150. Kim, J. H., T. M. Lewin, and R. A. Coleman. 2001. Expression and characterization of recombinant rat acyl-CoA synthetases 1, 4, and 5 - Selective inhibition by triacsin C and thiazolidinediones. *J. Biol. Chem.* **276**: 24667–24673.
151. Guo, Y., K. R. Cordes, R. V Farese Jr., and T. C. Walther. 2009. Lipid droplets at a glance. *J. Cell Sci.* **122**: 749–752.
152. Dvorak, A., P. Weller, V. Harvey, E. Morgan, and H. Dvorak. 1993. Ultrastructural-Localization of Prostaglandin Endoperoxide Synthase (Cyclooxygenase) to Isolated, Purified Fractions of Guinea-Pig Peritoneal Macrophage and Line 10 Hepatocarcinoma Cell Lipid Bodies. *Int. Arch. Allergy Immunol.* **101**: 136–142.
153. Accioly, M. T., P. Pacheco, C. M. Maya-Monteiro, N. Carrossini, B. K. Robbs, S. S. Oliveira, C. Kaufmann, J. A. Morgado-Diaz, P. T. Bozza, and J. P. Viola. 2008. Lipid bodies are reservoirs of cyclooxygenase-2 and sites of prostaglandin-E2 synthesis in colon cancer cells. *Cancer Res.* **68**: 1732–1740.
154. Chakrabarti, P., T. English, J. Shi, C. M. Smas, and K. V Kandror. 2010. Mammalian target of rapamycin complex 1 suppresses lipolysis, stimulates lipogenesis, and promotes fat storage. *Diabetes*. **59**: 775–781.
155. Maya-Monteiro, C. M., and P. T. Bozza. 2008. Leptin and mTOR: partners in metabolism and inflammation. *Cell Cycle*. **7**: 1713–1717.
156. Lu, X. Y., J. Gruia-Gray, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, C. Londos, and A. R. Kimmel. 2001. The murine perilipin gene: the lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin. *Mamm. Genome*. **12**:

741–749.
157. Brasaemle, D. L., and N. E. Wolins. 2012. Packaging of Fat: An Evolving Model of Lipid Droplet Assembly and Expansion. *J. Biol. Chem.* **287**: 2273–2279.
158. Guo, Y., T. C. Walther, M. Rao, N. Stuurman, G. Goshima, K. Terayama, J. S. Wong, R. D. Vale, P. Walter, and R. V Farese. 2008. Functional genomic screen reveals genes involved in lipid-droplet formation and utilization. *Nature*. **453**: 657–661.
159. Hsieh, K., Y. K. Lee, C. Londos, B. M. Raaka, K. T. Dalen, and A. R. Kimmel. 2012. Perilipin family members preferentially sequester to either triacylglycerol-specific or cholesteryl-ester-specific intracellular lipid storage droplets. *J. Cell Sci.* **125**: 4067–4076.
160. Kohjima, M., T. H. Tsai, B. C. Tackett, S. Thevananther, L. Li, B. H. Chang, and L. Chan. 2013. Delayed liver regeneration after partial hepatectomy in adipose differentiation related protein-null mice. *J. Hepatol.* **59**: 1246–1254.
161. Greco, D., A. Kotronen, J. Westerbacka, O. Puig, P. Arkkila, T. Kiviluoto, S. Laitinen, M. Kolak, R. M. Fisher, A. Hamsten, P. Auvinen, and H. Yki-Jarvinen. 2008. Gene expression in human NAFLD. *Am. J. Physiol. liver Physiol.* **294**: G1281-7.
162. Straub, B. K., P. Stoeffel, H. Heid, R. Zimbelmann, and P. Schirmacher. 2008. Differential pattern of lipid droplet-associated proteins and de novo perilipin expression in hepatocyte steatogenesis. *Hepatology*. **47**: 1936–1946.
163. Straub, B. K., E. Herpel, S. Singer, R. Zimbelmann, K. Breuhahn, S. Macher-Goeppinger, A. Warth, J. Lehmann-Koch, T. Longerich, H. Heid, and P. Schirmacher. 2010. Lipid droplet-associated PAT-proteins show frequent and differential expression in neoplastic steatogenesis. *Mod. Pathol.* **23**: 480–92. [online] http://www.ncbi.nlm.nih.gov/pubmed/20081801 (Accessed September 6, 2013).
164. Subramanian, V., A. Rothenberg, C. Gomez, A. W. Cohen, A. Garcia, S. Bhattacharyya, L. Shapiro, G. Dolios, R. Wang, M. P. Lisanti, and D. L. Brasaemle. 2004. Perilipin A mediates the reversible binding of CGI-58 to lipid droplets in 3T3-L1 adipocytes. *J. Biol. Chem.* **279**: 42062–42071.
165. Lass, A., R. Zimmermann, G. Haemmerle, M. Riederer, G. Schoiswohl, M. Schweiger, P. Kienesberger, J. G. Strauss, G. Gorkiewicz, and R. Zechner. 2006. Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. *Cell Metab.* **3**: 309–319.
166. Guo, F., Y. Ma, A. K. Kadegowda, J. L. Betters, P. Xie, G. Liu, X. Liu, H. Miao, J. Ou, X. Su, Z. Zheng, B. Xue, H. Shi, and L. Yu. 2013. Deficiency

of liver Comparative Gene Identification-58 causes steatohepatitis and fibrosis in mice. *J. Lipid Res.* **54**: 2109–2120.
167. Listenberger, L. L., A. G. Ostermeyer-Fay, E. B. Goldberg, W. J. Brown, and D. A. Brown. 2007. Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. *J. Lipid Res.* **48**: 2751–2761.
168. Stringer, D. M., P. Zahradka, V. C. DeClercq, N. R. Ryz, R. Diakiw, L. L. Burr, X. Xie, and C. G. Taylor. 2010. Modulation of lipid droplet size and lipid droplet proteins by trans-10,cis-12 conjugated linoleic acid parallels improvements in hepatic steatosis in obese, insulin-resistant rats. *Biochim. Biophys. Acta-Molecular Cell Biol. Lipids*. **1801**: 1375–1385.
169. McManaman, J. L., E. S. Bales, D. J. Orlicky, M. Jackman, P. S. MacLean, S. Cain, A. E. Crunk, A. Mansur, C. E. Graham, T. A. Bowman, and A. S. Greenberg. 2013. Perilipin-2-null mice are protected against diet-induced obesity, adipose inflammation, and fatty liver disease. *J. Lipid Res.* **54**: 1346–1359.
170. Carr, R. M., R. T. Patel, V. Rao, R. Dhir, M. J. Graham, R. M. Crooke, and R. S. Ahima. 2012. Reduction of TIP47 improves hepatic steatosis and glucose homeostasis in mice. *Am. J. Physiol. Integr. Comp. Physiol.* **302**: R996-1003.
171. Lee, J. S., R. Mendez, H. H. Heng, Z. Q. Yang, and K. Zhang. 2012. Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation. *Am. J. Transl. Res.* **4**: 102–113.
172. Liang, L., M. Zhao, Z. Xu, K. K. Yokoyama, and T. Li. 2003. Molecular cloning and characterization of CIDE-3, a novel member of the cell-death-inducing DNA-fragmentation-factor (DFF45)-like effector family. *Biochem. J.* **370**: 195–203.
173. LiZhen, W., Z. LinKang, C. Cheng, G. JingYi, X. Li, Y. Jing, L. De, and L. Peng. 2014. Cidea controls lipid droplet fusion and lipid storage in brown and white adipose tissue. *Sci. China-Life Sci.* **57**: 107–116.
174. Min, J., W. Zhang, Y. Gu, L. Hong, L. Yao, F. Li, D. Zhao, Y. Feng, H. Zhang, and Q. Li. 2011. CIDE-3 interacts with lipopolysaccharide-induced tumor necrosis factor, and overexpression increases apoptosis in hepatocellular carcinoma. *Med. Oncol.* **28**: S219–S227.
175. Sun, Z., J. Gong, H. Wu, W. Xu, L. Wu, D. Xu, J. Gao, J. W. Wu, H. Yang, M. Yang, and P. Li. 2013. Perilipin1 promotes unilocular lipid droplet formation through the activation of Fsp27 in adipocytes. *Nat. Commun.* **4**: 1594.
176. Yang, X., X. Lu, M. Lombes, G. B. Rha, Y. I. Chi, T. M. Guerin, E. J. Smart, and J. Liu. 2010. The G(0)/G(1) switch gene 2 regulates adipose lipolysis

through association with adipose triglyceride lipase. *Cell Metab.* **11**: 194–205.
177. Schweiger, M., M. Paar, C. Eder, J. Brandis, E. Moser, G. Gorkiewicz, S. Grond, F. P. Radner, I. Cerk, I. Cornaciu, M. Oberer, S. Kersten, R. Zechner, R. Zimmermann, and A. Lass. 2012. G0/G1 switch gene-2 regulates human adipocyte lipolysis by affecting activity and localization of adipose triglyceride lipase. *J. Lipid Res.* **53**: 2307–2317.
178. Zhang, W., I. O-Sullivan, T. G. Unterman, S. Bu, D. G. Mashek, and C. Kahn. 2012. Regulation of Adipose Triglyceride Lipase (Atgl) and Gos2 Lipid Droplet Proteins by Foxo Transcription Factors and Insulin in the Liver. *J. Investig. Med.* **60**: 724.
179. Yamada, T., C. S. Park, Y. Shen, K. R. Rabin, and H. D. Lacorazza. 2014. GOS2 inhibits the proliferation of K562 cells by interacting with nucleolin in the cytosol. *Leuk. Res.* **38**: 210–217.
180. Zhang, P., R. Wei, X. Wen, L. Ping, C. Wang, Z. Dong, X. Deng, W. Bo, C. Bin, and Y. Tian. 2012. Genes expression profiling of peripheral blood cells of patients with hepatocellular carcinoma. *Cell Biol. Int.* **36**: 803–809.
181. Chung, S. Y., J. M. Brown, M. B. Sandberg, and M. McIntosh. 2005. Trans-10,cis-12 CLA increases adipocyte lipolysis and alters lipid droplet-associated proteins: role of mTOR and ERK signaling. *J. Lipid Res.* **46**: 885–895.
182. Zhang, J., C. J. Hupfeld, S. S. Taylor, J. M. Olefsky, and R. Y. Tsien. 2005. Insulin disrupts beta-adrenergic signalling to protein kinase A in adipocytes. *Nature*. **437**: 569–573.
183. Greenberg, A. S., J. J. Egan, S. A. Wek, N. B. Garty, E. J. Blanchette-Mackie, and C. Londos. 1991. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J. Biol. Chem.* **266**: 11341–11346.
184. Fassett, J. T., D. Tobolt, C. J. Nelsen, J. H. Albrecht, and L. K. Hansen. 2003. The role of collagen structure in mitogen stimulation of ERK, cyclin D1 expression, and G1-S progression in rat hepatocytes. *J. Biol. Chem.* **278**: 31691–31700.
185. Weinberg, R. A. 2014. *In* The Biology of Cancer: Second Edition (Bochicchio, A., Zayatz, E., and Mickey, R. K., eds.). p. 275. , Garland Science, Taylor & Francis Group, LLC, New York, NY.
186. Ren, S., and B. J. Rollins. 2004. Cyclin C/cdk3 promotes Rb-dependent G0 exit. *Cell*. **117**: 239–251.
187. Wang, W., B. Bu, M. Xie, M. Zhang, Z. Yu, and D. Tao. 2009. Neural cell cycle dysregulation and central nervous system diseases. *Prog. Neurobiol.* **89**: 1–17.

188. Nelsen, C. J., D. G. Rickheim, M. M. Tucker, L. K. Hansen, and J. H. Albrecht. 2003. Evidence that cyclin D1 mediates both growth and proliferation downstream of TOR in hepatocytes. *J. Biol. Chem.* **278**: 3656–3663.
189. Watanabe, G., C. Albanese, R. J. Lee, A. Reutens, G. Vairo, B. Henglein, and R. G. Pestell. 1998. Inhibition of cyclin D1 kinase activity is associated with E2F-mediated inhibition of cyclin D1 promoter activity through E2F and Sp1. *Mol. Cell. Biol.* **18**: 3212–3222.
190. Kondo, T., S. Ezzat, and S. L. Asa. 2006. Pathogenetic mechanisms in thyroid follicular-cell neoplasia. *Nat. Rev. Cancer*. **6**: 292–306.
191. Alt, J. R., J. L. Cleveland, M. Hannink, and J. A. Diehl. 2000. Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes Dev.* **14**: 3102–3114.
192. Diehl, J. A., M. Cheng, M. F. Roussel, and C. J. Sherr. 1998. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* **12**: 3499–3511.
193. Sherr, C. J., and J. M. Roberts. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**: 1501–1512.
194. Hwang, H. C., and B. E. Clurman. 2005. Cyclin E in normal and neoplastic cell cycles. *Oncogene*. **24**: 2776–2786.
195. Nigg, E. A. 1995. Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *Bioessays*. **17**: 471–480.
196. Hershko, A. 1999. Mechanisms and regulation of the degradation of cyclin B. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **354**: 1571–1576.
197. Jung, K. Y., K. M. Kim, and S. Lim. 2014. Therapeutic Approaches for Preserving or Restoring Pancreatic beta-Cell Function and Mass. *Diabetes Metab. J.* **38**: 426–436.
198. Lee, Y., J. E. Dominy, Y. J. Choi, M. Jurczak, N. Tolliday, J. P. Camporez, H. Chim, J.-H. Lim, H.-B. Ruan, X. Yang, F. Vazquez, P. Sicinski, G. I. Shulman, and P. Puigserver. 2014. Cyclin D1-Cdk4 controls glucose metabolism independently of cell cycle progression. *Nature*. **510**: 547–551.
199. Nelsen, C. J., D. G. Rickheim, M. M. Tucker, T. J. McKenzie, L. K. Hansen, R. G. Pestell, and J. H. Albrecht. 2003. Amino acids regulate hepatocyte proliferation through modulation of cyclin D1 expression. *J. Biol. Chem.* **278**: 25853–25858.
200. Dorn, C., J. C. Engelmann, M. Saugspier, A. Koch, A. Hartmann, M. Muller, R. Spang, A. Bosserhoff, and C. Hellerbrand. 2014. Increased expression of c-Jun in nonalcoholic fatty liver disease. *Lab. Invest.* **94**: 394–408.
201. Mazza, A., B. Fruci, G. A. Garinis, S. Giuliano, R. Malaguarnera, and A. Belfiore. 2012. The role of metformin in the management of NAFLD. *Exp.*

Diabetes Res. **2012**: 716404.
202. Tsatsos, N. G., and H. C. Towle. 2006. Glucose activation of ChREBP in hepatocytes occurs via a two-step mechanism. *Biochem. Biophys. Res. Commun.* **340**: 449–456.
203. Stoeckman, A. K., L. Ma, and H. C. Towle. 2004. Mlx is the functional heteromeric partner of the carbohydrate response element-binding protein in glucose regulation of lipogenic enzyme genes. *J. Biol. Chem.* **279**: 15662–15669.
204. Davies, M. N., B. L. O'Callaghan, and H. C. Towle. 2008. Glucose activates ChREBP by increasing its rate of nuclear entry and relieving repression of its transcriptional activity. *J. Biol. Chem.* **283**: 24029–24038.
205. Hanse, E. A., D. G. Mashek, J. R. Becker, A. D. Solmonson, L. K. Mullany, M. T. Mashek, H. C. Towle, A. T. Chau, and J. H. Albrecht. 2012. Cyclin D1 inhibits hepatic lipogenesis via repression of carbohydrate response element binding protein and hepatocyte nuclear factor 4 alpha. *Cell Cycle*. **11**: 2681–2690.
206. Kamarajugadda, S., J. R. Becker, E. A. Hanse, D. G. Mashek, M. T. Mashek, A. M. Hendrickson, L. K. Mullany, and J. H. Albrecht. 2016. Cyclin D1 represses peroxisome proliferator-activated receptor alpha and inhibits fatty acid oxidation. *Oncotarget*. **7**: 47674–47686.
207. Sapiro, J. M., M. T. Mashek, A. S. Greenberg, and D. G. Mashek. 2009. Hepatic triacylglycerol hydrolysis regulates peroxisome proliferator-activated receptor alpha activity. *J. Lipid Res.* **50**: 1621–1629.
208. You, Q., M. Holt, H. Yin, G. Li, C.-J. Hu, and C. Ju. 2013. Role of hepatic resident and infiltrating macrophages in liver repair after acute injury. *Biochem. Pharmacol.* **86**: 836–843.
209. Cruise, J. L., K. A. Houck, and G. K. Michalopoulos. 1985. Induction of DNA synthesis in cultured rat hepatocytes through stimulation of alpha 1 adrenoreceptor by norepinephrine. *Science*. **227**: 749–751.
210. Block, G. D., J. Locker, W. C. Bowen, B. E. Petersen, S. Katyal, S. C. Strom, T. Riley, T. A. Howard, and G. K. Michalopoulos. 1996. Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium. *J. Cell Biol.* **132**: 1133–1149.
211. Nakagawa, S., L. Wei, W. M. Song, T. Higashi, S. Ghoshal, R. S. Kim, C. B. Bian, S. Yamada, X. Sun, A. Venkatesh, N. Goossens, G. Bain, G. Y. Lauwers, A. P. Koh, M. El-Abtah, N. B. Ahmad, H. Hoshida, D. J. Erstad, G. Gunasekaran, Y. Lee, M.-L. Yu, W.-L. Chuang, C.-Y. Dai, M. Kobayashi, H. Kumada, T. Beppu, H. Baba, M. Mahajan, V. D. Nair, M. Lanuti, A. Villanueva, A. Sangiovanni, M. Iavarone, M. Colombo, J. M.

Llovet, A. Subramanian, A. M. Tager, S. L. Friedman, T. F. Baumert, M. E. Schwarz, R. T. Chung, K. K. Tanabe, B. Zhang, B. C. Fuchs, and Y. Hoshida. 2016. Molecular Liver Cancer Prevention in Cirrhosis by Organ Transcriptome Analysis and Lysophosphatidic Acid Pathway Inhibition. *Cancer Cell*. **30**: 879–890.

212. Wu, R., Z. Chen, Y. Xu, J. Ge, and X. Lu. 2016. [Change in plasma nesfatin-1 concentration within high-fat diet induced nonalcoholic fatty liver disease rat models]. *Wei Sheng Yan Jiu*. **45**: 452–457.
213. Aguilar, V., and L. Fajas. 2010. Cycling through metabolism. *EMBO Mol. Med.* **2**: 338–348.
214. Chakrabarti, P., and K. V Kandror. 2009. FoxO1 Controls Insulin-dependent Adipose Triglyceride Lipase (ATGL) Expression and Lipolysis in Adipocytes. *J. Biol. Chem.* **284**: 13296–13300.
215. Haeusler, R. A., K. Hartil, B. Vaitheesvaran, I. Arrieta-Cruz, C. M. Knight, J. R. Cook, H. L. Kammoun, M. A. Febbraio, R. Gutierrez-Juarez, I. J. Kurland, and D. Accili. 2014. Integrated control of hepatic lipogenesis versus glucose production requires FoxO transcription factors. *Nat. Commun.* **5**: 5190.
216. Xiong, X., R. Tao, R. A. DePinho, and X. C. Dong. 2013. Deletion of hepatic FoxO1/3/4 genes in mice significantly impacts on glucose metabolism through downregulation of gluconeogenesis and upregulation of glycolysis. *PLoS One*. **8**: e74340.
217. Zhang, K., L. Li, Y. Qi, X. Zhu, B. Gan, R. A. DePinho, T. Averitt, and S. Guo. 2012. Hepatic suppression of Foxo1 and Foxo3 causes hypoglycemia and hyperlipidemia in mice. *Endocrinology*. **153**: 631–646.
218. Li, X., B. Monks, Q. Ge, and M. J. Birnbaum. 2007. Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator. *Nature*. **447**: 1012–1016.
219. Puigserver, P., J. Rhee, J. Donovan, C. J. Walkey, J. C. Yoon, F. Oriente, Y. Kitamura, J. Altomonte, H. J. Dong, D. Accili, and B. M. Spiegelman. 2003. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1 alpha interaction. *Nature*. **423**: 550–555.
220. Zhang, W., S. Y. Bu, M. T. Mashek, I. O-Sullivan, Z. Sibai, S. A. Khan, O. Ilkayeva, C. B. Newgard, D. G. Mashek, and T. G. Unterman. 2016. Integrated Regulation of Hepatic Lipid and Glucose Metabolism by Adipose Triacylglycerol Lipase and FoxO Proteins. *Cell Rep.* **15**: 349–359.
221. Coleman, R. A., and D. G. Mashek. 2011. Mammalian triacylglycerol metabolism: synthesis, lipolysis, and signaling. *Chem. Rev.* **111**: 6359–6386.
222. Zimmermann, R., J. G. Strauss, G. Haemmerle, G. Schoiswohl, R. Birner-

Gruenberger, M. Riederer, A. Lass, G. Neuberger, F. Eisenhaber, A. Hermetter, and R. Zechner. 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science (80-.).* **306**: 1383–1386.
223. Ong, K. T., M. T. Mashek, N. O. Davidson, and D. G. Mashek. 2014. Hepatic ATGL mediates PPAR-alpha signaling and fatty acid channeling through an L-FABP independent mechanism. *J. Lipid Res.* **55**: 808–815.
224. Khan, S. A., A. Sathyanarayan, M. T. Mashek, K. T. Ong, E. E. Wollaston-Hayden, and D. G. Mashek. 2015. ATGL-catalyzed lipolysis regulates SIRT1 to control PGC-1alpha/PPAR-alpha signaling. *Diabetes*. **64**: 418–426.
225. Das, S. K., and G. Hoefler. 2013. The role of triglyceride lipases in cancer associated cachexia. *Trends Mol. Med.* **19**: 292–301.
226. Seton-Rogers, S. 2011. Cachexia: Putting the brakes on lipid loss. *Nat. Rev. Cancer*. **11**: 536.
227. Mullins, G. R., L. Wang, V. Raje, S. G. Sherwood, R. C. Grande, S. Boroda, J. M. Eaton, S. Blancquaert, P. P. Roger, N. Leitinger, and T. E. Harris. 2014. Catecholamine-induced lipolysis causes mTOR complex dissociation and inhibits glucose uptake in adipocytes. *Proc. Natl. Acad. Sci. U. S. A.* **111**: 17450–17455.
228. Pauta, M., N. Rotllan, A. Fernandez-Hernando, C. Langhi, J. Ribera, M. Lu, L. Boix, J. Bruix, W. Jimenez, Y. Suarez, D. A. Ford, A. Baldan, M. J. Birnbaum, M. Morales-Ruiz, and C. Fernandez-Hernando. 2016. Akt-mediated foxo1 inhibition is required for liver regeneration. *Hepatology*. **63**: 1660–1674.
229. Walther, T. C., and R. V. J. Farese. 2012. Lipid droplets and cellular lipid metabolism. *Annu. Rev. Biochem.* **81**: 687–714.
230. Michalopoulos, G. K. 2007. Liver regeneration. *J. Cell. Physiol.* **213**: 286–300.
231. Lord, C. C., D. Ferguson, G. Thomas, A. L. Brown, R. C. Schugar, A. Burrows, A. D. Gromovsky, J. Betters, C. Neumann, J. Sacks, S. Marshall, R. Watts, M. Schweiger, R. G. Lee, R. M. Crooke, M. J. Graham, J. D. Lathia, T. F. Sakaguchi, R. Lehner, G. Haemmerle, R. Zechner, and J. M. Brown. 2016. Regulation of Hepatic Triacylglycerol Metabolism by CGI-58 Does Not Require ATGL Co-activation. *Cell Rep.* **16**: 939–949.
232. Laplante, M., and D. M. Sabatini. 2012. mTOR signaling in growth control and disease. *Cell*. **149**: 274–93.
233. Hanse, E. A., D. G. Mashek, J. R. Becker, A. D. Solmonson, L. K. Mullany, M. T. Mashek, H. C. Towle, A. T. Chau, and J. H. Albrecht. 2012. Cyclin D1 inhibits hepatic lipogenesis via repression of carbohydrate response element binding protein and hepatocyte nuclear factor 4alpha. *Cell Cycle*.

11: 2681–2690.
234. Fujii, H., Y. Ikura, J. Arimoto, K. Sugioka, J. C. Iezzoni, S. H. Park, T. Naruko, H. Itabe, N. Kawada, S. H. Caldwell, and M. Ueda. 2009. Expression of perilipin and adipophilin in nonalcoholic fatty liver disease; relevance to oxidative injury and hepatocyte ballooning. *J. Atheroscler. Thromb.* **16**: 893–901.
235. Imai, Y., G. M. Varela, M. B. Jackson, M. J. Graham, R. M. Crooke, and R. S. Ahima. 2007. Reduction of hepatosteatosis and lipid levels by an adipose differentiation-related protein antisense oligonucleotide. *Gastroenterology*. **132**: 1947–1954.
236. Younossi, Z. M., M. Otgonsuren, L. Henry, C. Venkatesan, A. Mishra, M. Erario, and S. Hunt. 2015. Association of nonalcoholic fatty liver disease (NAFLD) with hepatocellular carcinoma (HCC) in the United States from 2004 to 2009. *Hepatology*. **62**: 1723–1730.
237. Ryan, M. C., C. Itsiopoulos, T. Thodis, G. Ward, N. Trost, S. Hofferberth, K. O'Dea, P. V Desmond, N. A. Johnson, and A. M. Wilson. 2013. The Mediterranean diet improves hepatic steatosis and insulin sensitivity in individuals with non-alcoholic fatty liver disease. *J. Hepatol.* **59**: 138–143.
238. Parkinson, L., and S. Cicerale. 2016. The Health Benefiting Mechanisms of Virgin Olive Oil Phenolic Compounds. *Molecules*. **21**.
239. Jurado-Ruiz, E., L. M. Varela, A. Luque, G. Berna, G. Cahuana, E. Martinez-Force, R. Gallego-Duran, B. Soria, B. de Roos, M. Romero Gomez, and F. Martin. 2017. An extra virgin olive oil rich diet intervention ameliorates the nonalcoholic steatohepatitis induced by a high-fat "Western-type" diet in mice. *Mol. Nutr. Food Res.* **61**.
240. Lee, I. M., C. C. Hsieh, and R. S. J. Paffenbarger. 1995. Exercise intensity and longevity in men. The Harvard Alumni Health Study. *JAMA*. **273**: 1179–1184.
241. Meka, N., S. Katragadda, B. Cherian, and R. R. Arora. 2008. Endurance exercise and resistance training in cardiovascular disease. *Ther. Adv. Cardiovasc. Dis.* **2**: 115–121.
242. Willis, L. H., C. A. Slentz, L. A. Bateman, A. T. Shields, L. W. Piner, C. W. Bales, J. A. Houmard, and W. E. Kraus. 2012. Effects of aerobic and/or resistance training on body mass and fat mass in overweight or obese adults. *J. Appl. Physiol.* **113**: 1831–1837.
243. Lee, I. M., and R. S. J. Paffenbarger. 2000. Associations of light, moderate, and vigorous intensity physical activity with longevity. The Harvard Alumni Health Study. *Am. J. Epidemiol.* **151**: 293–299.
244. Piguet, A.-C., U. Saran, C. Simillion, I. Keller, L. Terracciano, H. L. Reeves, and J.-F. Dufour. 2015. Regular exercise decreases liver tumors

development in hepatocyte-specific PTEN-deficient mice independently of steatosis. *J. Hepatol.* **62**: 1296–1303.
245. Belahsen, R., and M. Rguibi. 2006. Population health and Mediterranean diet in southern Mediterranean countries. *Public Health Nutr.* **9**: 1130–1135.
246. Rocha, N. S., L. F. Barbisan, M. L. C. de Oliveira, and J. L. V. de Camargo. 2002. Effects of fasting and intermittent fasting on rat hepatocarcinogenesis induced by diethylnitrosamine. *Teratog. Carcinog. Mutagen.* **22**: 129–138.
247. Dunlap, S. M., L. J. Chiao, L. Nogueira, J. Usary, C. M. Perou, L. Varticovski, and S. D. Hursting. 2012. Dietary energy balance modulates epithelial-to-mesenchymal transition and tumor progression in murine claudin-low and basal-like mammary tumor models. *Cancer Prev. Res. (Phila).* **5**: 930–942.
248. Galet, C., A. Gray, J. W. Said, B. Castor, J. Wan, P. J. Beltran, F. J. Calzone, D. Elashoff, P. Cohen, and W. J. Aronson. 2013. Effects of calorie restriction and IGF-1 receptor blockade on the progression of 22Rv1 prostate cancer xenografts. *Int. J. Mol. Sci.* **14**: 13782–13795.
249. Jiang, Y.-S., and F.-R. Wang. 2013. Caloric restriction reduces edema and prolongs survival in a mouse glioma model. *J. Neurooncol.* **114**: 25–32.
250. Harvey, A. E., L. M. Lashinger, G. Otto, N. P. Nunez, and S. D. Hursting. 2013. Decreased systemic IGF-1 in response to calorie restriction modulates murine tumor cell growth, nuclear factor-kappaB activation, and inflammation-related gene expression. *Mol. Carcinog.* **52**: 997–1006.
251. Lanza-Jacoby, S., G. Yan, G. Radice, C. LePhong, J. Baliff, and R. Hess. 2013. Calorie restriction delays the progression of lesions to pancreatic cancer in the LSL-KrasG12D; Pdx-1/Cre mouse model of pancreatic cancer. *Exp. Biol. Med. (Maywood).* **238**: 787–797.
252. Kim, K. E., Y. Jung, S. Min, M. Nam, R. W. Heo, B. T. Jeon, D. H. Song, C.-O. Yi, E. A. Jeong, H. Kim, J. Kim, S.-Y. Jeong, W. Kwak, D. H. Ryu, T. L. Horvath, G. S. Roh, and G.-S. Hwang. 2016. Caloric restriction of db/db mice reverts hepatic steatosis and body weight with divergent hepatic metabolism. *Sci. Rep.* **6**: 30111.
253. McCay, C. M., M. F. Crowell, and L. A. Maynard. 1989. The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. *Nutrition*. **5**: 155–71; discussion 172.
254. Vandbakk, K., B. Welde, A. H. Kruken, J. Baumgart, G. Ettema, T. Karlsen, and O. Sandbakk. 2017. Effects of upper-body sprint-interval training on strength and endurance capacities in female cross-country skiers. *PLoS One*. **12**: e0172706.

255. Nakagawa, H. 2015. Recent advances in mouse models of obesity- and nonalcoholic steatohepatitis-associated hepatocarcinogenesis. *World J. Hepatol.* **7**: 2110–2118.
256. Gong, Z., E. Tas, S. Yakar, and R. Muzumdar. 2016. Hepatic lipid metabolism and non-alcoholic fatty liver disease in aging. *Mol. Cell. Endocrinol.*
257. Guan, L., L. Zhang, Z. Gong, X. Hou, Y. Xu, X. Feng, H. Wang, and H. You. 2016. FoxO3 inactivation promotes human cholangiocarcinoma tumorigenesis and chemoresistance through Keap1-Nrf2 signaling. *Hepatology*. **63**: 1914–1927.
258. McCommis, K. S., W. T. Hodges, E. M. Brunt, Ilk. Nalbantoglu, W. G. McDonald, C. Holley, H. Fujiwara, J. E. Schaffer, J. R. Colca, and B. N. Finck. 2016. Targeting the Mitochondrial Pyruvate Carrier Attenuates Fibrosis in a Mouse Model of Nonalcoholic Steatohepatitis. *Hepatology*.
259. Wang, Q., W.-N. Yu, X. Chen, X. Peng, S.-M. Jeon, M. J. Birnbaum, G. Guzman, and N. Hay. 2016. Spontaneous Hepatocellular Carcinoma after the Combined Deletion of Akt Isoforms. *Cancer Cell*. **29**: 523–535.
260. Spindler, S. R. 2005. Rapid and reversible induction of the longevity, anticancer and genomic effects of caloric restriction. *Mech. Ageing Dev.* **126**: 960–966.
261. Colman, R. J., R. M. Anderson, S. C. Johnson, E. K. Kastman, K. J. Kosmatka, T. M. Beasley, D. B. Allison, C. Cruzen, H. A. Simmons, J. W. Kemnitz, and R. Weindruch. 2009. Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science*. **325**: 201–204.
262. Lv, M., X. Zhu, H. Wang, F. Wang, and W. Guan. 2014. Roles of caloric restriction, ketogenic diet and intermittent fasting during initiation, progression and metastasis of cancer in animal models: a systematic review and meta-analysis. *PLoS One*. **9**: e115147.
263. Rector, R. S., G. M. Uptergrove, E. M. Morris, S. J. Borengasser, M. H. Laughlin, F. W. Booth, J. P. Thyfault, and J. A. Ibdah. 2011. Daily exercise vs. caloric restriction for prevention of nonalcoholic fatty liver disease in the OLETF rat model. *Am. J. Physiol. Gastrointest. Liver Physiol.* **300**: G874-83.
264. Holmberg, S. D., P. R. Spradling, A. C. Moorman, and M. M. Denniston. 2013. Hepatitis C in the United States. *N. Engl. J. Med.* **368**: 1859–1861.
265. Han, J., and R. J. Ulevitch. 2005. Limiting inflammatory responses during activation of innate immunity. *Nat. Immunol.* **6**: 1198–1205.
266. Meyer-Bahlburg, A., S. Khim, and D. J. Rawlings. 2007. B cell intrinsic TLR signals amplify but are not required for humoral immunity. *J. Exp. Med.* **204**: 3095–3101.

267. Borlak, J., F. Langer, R. Spanel, G. Schondorfer, and C. Dittrich. 2016. Immune-mediated liver injury of the cancer therapeutic antibody catumaxomab targeting EpCAM, CD3 and Fcgamma receptors. *Oncotarget*. **7**: 28059–28074.
268. Dalpke, A. H., M. D. Lehner, T. Hartung, and K. Heeg. 2005. Differential effects of CpG-DNA in Toll-like receptor-2/-4/-9 tolerance and cross-tolerance. *Immunology*. **116**: 203–212.
269. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*. **413**: 732–738.
270. Seki, E., S. De Minicis, C. H. Osterreicher, J. Kluwe, Y. Osawa, D. A. Brenner, and R. F. Schwabe. 2007. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat. Med.* **13**: 1324–1332.
271. Seki, E., and D. A. Brenner. 2008. Toll-like receptors and adaptor molecules in liver disease: update. *Hepatology*. **48**: 322–335.
272. Watanabe, A., A. Hashmi, D. A. Gomes, T. Town, A. Badou, R. A. Flavell, and W. Z. Mehal. 2007. Apoptotic hepatocyte DNA inhibits hepatic stellate cell chemotaxis via toll-like receptor 9. *Hepatology*. **46**: 1509–1518.
273. Xu, X., Y.-C. Ning, W. Wang, J. Liu, X. Bai, X. Sun, G. Cai, and X. Chen. 2015. Anti-Inflamm-Aging Effects of Long-Term Caloric Restriction via Overexpression of SIGIRR to Inhibit NF-kappaB Signaling Pathway. *Cell. Physiol. Biochem.* **37**: 1257–1270.
274. Lee, I. S., G. Shin, and R. Choue. 2010. A 12-week regimen of caloric restriction improves levels of adipokines and pro-inflammatory cytokines in Korean women with BMIs greater than 23 kg/m2. *Inflamm. Res.* **59**: 399–405.
275. Fuchs, B. C., Y. Hoshida, T. Fujii, L. Wei, S. Yamada, G. Y. Lauwers, C. M. McGinn, D. K. DePeralta, X. Chen, T. Kuroda, M. Lanuti, A. D. Schmitt, S. Gupta, A. Crenshaw, R. Onofrio, B. Taylor, W. Winckler, N. Bardeesy, P. Caravan, T. R. Golub, and K. K. Tanabe. 2014. Epidermal growth factor receptor inhibition attenuates liver fibrosis and development of hepatocellular carcinoma. *Hepatology*. **59**: 1577–1590.
276. Zhang, Y., N. Xu, J. Xu, B. Kong, B. Copple, G. L. Guo, and L. Wang. 2014. E2F1 is a novel fibrogenic gene that regulates cholestatic liver fibrosis through the Egr-1/SHP/EID1 network. *Hepatology*. **60**: 919–930.
277. Okamura, Y., S. Nomoto, M. Kanda, Q. Li, Y. Nishikawa, H. Sugimoto, N. Kanazumi, S. Takeda, and A. Nakao. 2010. Leukemia inhibitory factor receptor (LIFR) is detected as a novel suppressor gene of hepatocellular carcinoma using double-combination array. *Cancer Lett.* **289**: 170–177.
278. Yang, Z. F., D. W. Ho, M. N. Ng, C. K. Lau, W. C. Yu, P. Ngai, P. W. K.

Chu, C. T. Lam, R. T. P. Poon, and S. T. Fan. 2008. Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell*. **13**: 153–166.
279. Lama, A., C. Pirozzi, M. P. Mollica, G. Trinchese, F. Di Guida, G. Cavaliere, A. Calignano, G. Mattace Raso, R. Berni Canani, and R. Meli. 2017. Polyphenol-rich virgin olive oil reduces insulin resistance and liver inflammation and improves mitochondrial dysfunction in high-fat diet fed rats. *Mol. Nutr. Food Res.* **61**.
280. Meijer, J. H., and Y. Robbers. 2014. Wheel running in the wild. *Proceedings. Biol. Sci.* **281**.
281. Morton, G. J., K. J. Kaiyala, J. D. Fisher, K. Ogimoto, M. W. Schwartz, and B. E. Wisse. 2011. Identification of a physiological role for leptin in the regulation of ambulatory activity and wheel running in mice. *Am. J. Physiol. Endocrinol. Metab.* **300**: E392-401.
282. Franco, S., V. Freid, J. Holmes, M. Bush, L. Curl, V. Freid, N. Han, and H. Khajuria. 2016. Health, United States, 2015. Hyattsville.
283. Cawley, J., and C. Meyerhoefer. 2012. The medical care costs of obesity: an instrumental variables approach. *J. Health Econ.* **31**: 219–230.
284. Bomberg, E., L. Birch, N. Endenburg, A. J. German, J. Neilson, H. Seligman, G. Takashima, and M. J. Day. 2017. The Financial Costs, Behaviour and Psychology of Obesity: A One Health Analysis. *J. Comp. Pathol.* **156**: 310–325.
285. Zhou, X., J. Yu, L. Li, V. L. Gloy, A. Nordmann, M. Tiboni, Y. Li, and X. Sun. 2016. Effects of Bariatric Surgery on Mortality, Cardiovascular Events, and Cancer Outcomes in Obese Patients: Systematic Review and Meta-analysis. *Obes. Surg.* **26**: 2590–2601.
286. El-Serag, H. B. 2011. Hepatocellular carcinoma. *N. Engl. J. Med.* **365**: 1118–1127.
287. Mazzanti, R. 2016. Hepatocellular carcinoma: Where are we? *World J. Exp. Med.* **6**: 21.
288. El-Serag, H. B., and J. A. Davila. 2011. Surveillance for hepatocellular carcinoma: in whom and how? *Therap. Adv. Gastroenterol.* **4**: 5–10.
289. Summa, K. C., and F. W. Turek. 2014. Chronobiology and obesity: Interactions between circadian rhythms and energy regulation. *Adv. Nutr.* **5**: 312S–9S.
290. Huang, W., K. M. Ramsey, B. Marcheva, and J. Bass. 2011. Circadian rhythms, sleep, and metabolism. *J. Clin. Invest.* **121**: 2133–2141.
291. Kettner, N. M., H. Voicu, M. J. Finegold, C. Coarfa, A. Sreekumar, N. Putluri, C. A. Katchy, C. Lee, D. D. Moore, and L. Fu. 2016. Circadian Homeostasis of Liver Metabolism Suppresses Hepatocarcinogenesis. *Cancer Cell*. **30**: 909–924.

292. Heymsfield, S. B., J. B. Harp, M. L. Reitman, J. W. Beetsch, D. A. Schoeller, N. Erondu, and A. Pietrobelli. 2007. Why do obese patients not lose more weight when treated with low-calorie diets? A mechanistic perspective. *Am. J. Clin. Nutr.* **85**: 346–354.
293. Vander Heiden, M., L. C. Cantley, and C. B. Thompson. 2009. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science (80-.).* **324**: 1029–1033.
294. Das, S. K., S. Eder, S. Schauer, C. Diwoky, H. Temmel, B. Guertl, G. Gorkiewicz, K. P. Tamilarasan, P. Kumari, M. Trauner, R. Zimmermann, P. Vesely, G. Haemmerle, R. Zechner, and G. Hoefler. 2011. Adipose triglyceride lipase contributes to cancer-associated cachexia. *Science*. **333**: 233–238.
295. Matter, M. S., T. Decaens, J. B. Andersen, and S. S. Thorgeirsson. 2014. Targeting the mTOR pathway in hepatocellular carcinoma: current state and future trends. *J. Hepatol.* **60**: 855–865.
296. Hu, B., M. Sun, J. Liu, G. Hong, and Q. Lin. 2016. The preventative effect of Akt knockout on liver cancer through modulating NF-kappaB-regulated inflammation and Bad-related apoptosis signaling pathway. *Int. J. Oncol.* **48**: 1467–1476.
297. Ong, K. T., M. T. Mashek, S. Y. Bu, and D. G. Mashek. 2013. Hepatic ATGL knockdown uncouples glucose intolerance from liver TAG accumulation. *Faseb J.* **27**: 313–321.
298. Tan, C., S. Liu, S. Tan, X. Zeng, H. Yu, A. Li, C. Bei, and X. Qiu. 2015. Polymorphisms in microRNA target sites of forkhead box O genes are associated with hepatocellular carcinoma. *PLoS One*. **10**: e0119210.
299. Sangodkar, J., N. S. Dhawan, H. Melville, V. J. Singh, E. Yuan, H. Rana, S. Izadmehr, C. Farrington, S. Mazhar, S. Katz, T. Albano, P. Arnovitz, R. Okrent, M. Ohlmeyer, M. Galsky, D. Burstein, D. Zhang, K. Politi, A. Difeo, and G. Narla. 2012. Targeting the FOXO1/KLF6 axis regulates EGFR signaling and treatment response. *J. Clin. Invest.* **122**: 2637–2651.
300. Huang, H., and D. J. Tindall. 2007. Dynamic FoxO transcription factors. *J. Cell Sci.* **120**: 2479–2487.